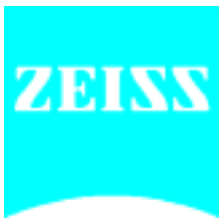
LSM 510
Laser Scanning Microscope

Operating Manual



I

Knowledge of this manual is required for the operation of the instrument. Would you therefore please make yourself familiar with the contents of this manual and pay special attention to hints concerning the safe operation of the instrument.

The specifications are subject to change; the manual is not covered by an update service.

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How to make best use of the LSM 510 operating instructions

A few symbols in these operating instructions will help you to recognize the nature and purpose of information immediately:



The WARNING symbol warns against hazards for the user that might arise when operating the laser.



This WARNING symbol warns against hazards from dangerously high voltages.



The CAUTION symbol warns against faults and hazards that might arise during operation and which might cause damage to the unit.



The NOTE symbol will help you to optimally solve your work problem. It represents a practical tip which will help you to find out which settings and methods are capable of improving or accelerating a procedure.



The HOT SURFACE symbol warns against hazards for the user that might arise when touching the lamp housing during operation.



The MAINS PLUG symbol remembers service personal to pull the mains plug before opening the device housing.

Depending on the problem, these operating instructions will supply you with various possibilities:

- If you want to know where to find certain general areas of information, refer to the following outline of sections to get a general overview.
- You will find a detailed table of contents at the start of every chapter. There you will see at a glance what topics are dealt with in detail.

Always remember: The time you invest in getting acquainted with the product will pay for itself many times over in your application task.

Scope of Equipment Supplied

Country: .....
Order number: .....
Serial number: .....
Delivery date: .....
Custom configuration: .....

Axioplan 2 MOT	000000-1028-778	☐
Axiovert 100 M SP	000000-1028-779	☐
Axiovert 100 M BP	000000-1028-780	☐

Objectives: .....
.....
.....

Confocal Laser Scanning Module LSM 510

Configuration 1	000000-1027-076	☐
Configuration 2	000000-1027-077	☐
Configuration 3	000000-1027-078	☐
Configuration 4	000000-1027-079	☐
Configuration 5	000000-1027-080	☐
Configuration 6	000000-1027-081	☐
Configuration 7	000000-1027-082	☐
Configuration 8	000000-1027-083	☐
Configuration 9	000000-1027-084	☐
Configuration 10	000000-1027-085	☐
Configuration 11	000000-1027-086	☐
Configuration 12	000000-1027-087	☐
Configuration 13	000000-1027-088	☐
Configuration 14	000000-1031-725	☐
Configuration 15	000000-1031-726	☐
Configuration 16	000000-1031-727	☐

Control computer with 21“ monitor	000000-1032-142	☐
Control computer with two 21“ monitors	000000-1032-143	☐

The license to the LSM control software is included in each configuration 1...16.

Optional software;

LSM 510 basis software release 2.0	000000-1027-554	☐
LSM 510 evaluation software physiology	000000-1027-556	☐
LSM 510 software 3D for LSM	000000-1024-966	☐

Kodak XLS 8650 PS printer	417735 9003-000	☐
Transparency exposure device (D)	412410 9011-000	☐
Large system table	453031-0000-000	☐
Small sytem table	453032-0000-000	☐
System baseplate	453030-0101-000	☐
UV laser	412410 9015-000	☐
High resolution z-stage HRZ 200 for Axiovert	000000-1013-186	☐
High resolution z-stage HRZ 200 for Axioplan 2	000000-1013-187	☐
XY scanning stage for Axiovert 100 M BP	000000-1017-917	☐
XY scanning stage for Axioplan 2 MOT	000000-1027-823	☐
Filter changer 18 mm	453070-0000-000	☐
Set of INDO filters	447960-0000-000	☐
Set of SNARF filters	447961-0000-000	☐

The LSM 510 in the configuration as checked above

was installed and handed to the customer in functional condition

on .....

by .....

Phone: .....

Fax: .....

The customer has been instructed on how to operate and maintain the equipment.

(Place)....., (date) .....

.....
Carl Zeiss Jena GmbH
Microscopy Division

.....
Customer

One copy to be kept by customer
One copy to be kept by Carl Zeiss

1 This section contains general notes on device safety, safe operation, and possible hazards caused by failure to observe the instructions.

2 The Setup Requirements section outlines the installation and supply requirements of the LSM 510 Microscope System, together with the relevant specifications.

3 Here you will find an introduction to Laser Scanning Microscopy, with an explanation of the principles of confocal imaging. The section also outlines the ways to present LSM image series in three dimensions, and introduces you to the performance features of your LSM 510.

4 In the Operation section you will find the most important steps and procedures of the LSM menu structure.The step-by-step description how to get an image will be shown by typical application examples including the WINDOWS NT 4.0 graphic user environment.

5 This section contains a description of the LSM 3D software package (basic program and add-ons. At the same time, all functions and settings are presented in a systematic form and in order in which they can be reached from the basic menu via sub-menus and dialog boxes

.

1 Notes on Device Safety

2 LSM 510 Setup Requirements

3 Introduction to Laser Scanning Microscopy

4 Operation

5 Software 3D Description

CHAPTER 1 NOTES ON DEVICE SAFETY

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1.3 Notes on setting up the microscope system 1-4

1.4 Notes on handling the computer and data media 1-5

1.5 Notes on care, maintenance and service 1-6

1.6 Notes on handling the laser components 1-7

1.7 Warning and information labels 1-7

1 NOTES ON DEVICE SAFETY

1.1 General

The LSM 510 laser scanning microscope, including its original accessories and compatible accessories from other manufacturers, may only be used for the purposes and microscopy techniques described in this manual (intended use).



The manufacturer will not assume liability for any malfunction or damage caused by any thing other than the intended use of the LSM 510 or individual modules or parts of it, nor by any repair or other service operation performed or attempted by persons other than duly authorized service staff. Any such action will invalidate any claim under warranty, including parts not directly affected by such action.

1.2 Regulations

Extensive knowledge of the hardware/the system is indispensable for safe operation of the LSM 510.



Read these operating instructions and all device publications belonging to the system conscientiously **before** operating the LSM 510! You can obtain additional information on the hardware configuration delivered and on optional system extensions from the manufacturer or via the service hotline.

⇒ The LSM 510 has been designed, built and tested in conformity with the standards DIN EN 61010-1 (IEC 1010-1) "Safety requirements for electrical instrumentation and control and laboratory apparatus", and DIN EN 60825-1 (IEC publication 825-1) "Safety of laser equipment", and taking relevant CSA and UL specifications into account.

⇒ As the system is largely operated via menus on a computer, you should be acquainted with the principles of the operating system and its WINDOWS NT 4.0 graphical user interface. The respective manuals are supplied together with the programs.

⇒ In accordance with WHO regulations, the LSM 510 is a device that belongs to laser hazard class 3 B. WHO recommendations concerning health and industrial protection when handling laser devices must be observed. The operator of the unit must also observe all and any relevant statutory accident prevention regulations.

1.3 Notes on setting up the microscope system

☞ Setting up, assembly on the system base plate and commissioning of the LSM 510 must be performed by authorized Carl Zeiss service staff, who are also advised to give the customer's operators a basic introduction to operation and maintenance.

The LSM 510 laser scanning microscope is delivered in several crates:

- Crate 1: microscope stand, laser scanning module, control unit
- Crate 2: computer
- Crate 3: monitor
- Crate 4: large system table
- Crate 5: second microscope stand
- Crate 6: small system table

⚠ The LSM 510 must be set up so as to ensure that the minimum clearance between the wall and the rear of the system is no less than 0.5 m. This clearance is needed for adjustment and maintenance operations.

Do not set up the unit in the proximity of heat sources such as radiators or direct sunlight. To avoid heat build-ups, the ventilation louvers on the microscope system must not be covered up.

The unit must be connected to a properly installed socket outlet with earthing contact by means of the mains cables supplied. Continuity of PE connection must not be affected by the use of extension leads.

⚠ Before connecting the mains cables, please check whether your mains voltage corresponds to the voltage specified on the rating plate of the laser module.

⚠ Maintenance, repairs, modifications, removal or exchange of components, or other interference with the equipment beyond the operations described in this manual may only be carried out by the manufacturer Carl Zeiss or by persons expressly authorized by us to do so. This applies especially to the microscope system, the laser scanning module, lasers, the PC system, the power supply units, cable connections and other system components.
Please note that the LSM 510 is a high-precision opto-electronic instrument. Inexpert handling may easily impair it's function or even damage it.

After installation or after conversion of the LSM system, authorized specialized staff must carefully check that it is in a proper condition and, particularly, that covers protecting against laser radiation are provided.

Tube openings or other unused mounts should always be protected against dust and moisture with the corresponding device components or with termination covers/blind plugs.

By establishing a corresponding workplace environment, please ensure that the formation of electrostatic charges by electronic components is avoided.
To avoid vibrations during operation, the LSM 510 should only be operated in conjunction with the system table (vibration damping).

1.4 Notes on handling the computer and data media

The computer used as standard in your LSM system is an IBM-compatible high-end pentium computer with WINDOWS NT (Version 4.0) operating system.

As standard, your computer has one hard disk drive, one drive for 1.44 MB diskettes and one CD-ROM drive. An additional 640 MB 3.5" MOD drive is installed.

☞ Do make sure, though, that you receive your LSM system with the operating system installed, with initialization and start files set up and with the LSM program also installed.

☞ When working with the hard disk, it is important to know that the more data it contains, the slower its operation will become. Therefore, data that you do not need permanently should be stored on a diskette or MOD.

⚠ When handling diskettes, avoid data losses by protecting them against extreme temperatures, moisture and magnetic fields. The data on a diskette is stored in the form of magnetic signals. To some extent, monitors, telephones or even lamps generate magnetic fields that might destroy this data. Also, never open the metal cover on diskette cases. A diskette´s surface can also be destroyed by touching it.

⚠ Never turn your computer off before you have exited the LSM program and run down the WINDOWS NT operating system. Otherwise, the program and/or data files may get lost.

⚠ When handling discs of the CD-ROM reader, do not touch the data side of the disc (the side of the disc with no label or printing).
Do not apply paper labels or write on any part of the disc, data side or label side. If dust or fingerprints get on the disc, wipe it with a soft cloth from the center to the edge, but do not use benzine, paint thinner, record cleaner, or static repellent. This can damage the disc.
Do not place the disc in any place where it is exposed to direct sunlight or high temperatures.

1.5 Notes on care, maintenance and service

The manufacturer of the unit cannot be held liable for damage resulting from operating errors, negligence or unauthorized tampering with the device system, particularly as the result of removal or replacement of parts of the unit or as the result of the use of unsuitable accessories from other manufacturers.
Any such action will also render all warranty claims null and void.

You are well advised to arrange a service agreement with your nearest Zeiss representative to guarantee perfect functioning of the microscope system in the long term.

Modifications and conversion work on the components of the system must only be carried out by the manufacturer, by the service agency or by persons authorized and trained for this purpose by the manufacturer.

Damaged units or parts may only be repaired or maintained by the responsible service agency.

Care operations that may be carried out by operating staff are limited to cleaning painted and glass surfaces.

- Cleaning painted surfaces
  To do this, use a clean cloth that has been moistened in a mixture of water and some detergent; do not use any solvent, however. Dry with a lint-free cloth.
- Cleaning glass surfaces
  Glass surfaces that have become soiled or which are marked with fingerprints may be rubbed with a clean optical cleaning cloth.
  If soiling is persistent, dip the optical cleaning cloth into a mixture of distilled water and a small quantity of detergent.
  To complete cleaning, lightly breathe on the glass surface and rub it dry with a clean cloth. Lint or dust is best removed with a clean hairbrush.

The air filter mat at the bottom of the LSM 510 Control Unit must be cleaned every six months. Filter mats can be ordered from our Service Department.

1.6 Notes on handling the laser components



The LSM 510 is a laser hazard class 3 B instrument and is marked as such.
This moderate-risk class embraces medium-power lasers. You must take care not to expose yourself to the radiation of such lasers. In particular, never look into the laser beam!

The following laser types are currently recommended for use in the LSM 510.

1 Ar 351/364 (UV)
2 Ar 488
3 Ar/ML 458/488/514
4 HeNe 543
5 ArKr 488/568
6 HeNe 633

☞ Please contact Carl Zeiss if you intend to use a laser type with a wavelength other than the ones above.

If used properly, the LSM 510 will not pose any laser radiation risks for operating staff. The dangerous laser radiation area is limited to the beam path and to a distance of up to around 10 cm from the specimen. Nevertheless, you should observe the following warnings:



- If necessary - insofar as specified by law - inform the laser protection officer before commissioning the laser.
- Always store laser key switches and, if applicable, keys for further laser power supply units, where they are inaccessible to persons not authorized to operate the laser.
- Do not place any reflecting objects into the beam path.
- Never open any covers or panelings.
- Never look into the laser beam, not even to simply view the specimen, whether with the aid of optical instruments or without. **Otherwise you risk going blind!**
- Do not leave any srew positions of the nosepiece uncovered.

☞ Suitable protective measures must be taken if gases, dust or vapors hazardous to health, secondary radiation or explosive objects should arise on the specimen as a result of laser radiation.

1.7 Warning and information labels

The warning and information labels attached on the LSM 510 must be observed. Check whether all of the labels shown below are provided on your instrument, and contact Carl Zeiss Germany or one of the service agencies if you should discover that any of the labels should be missing. You will receive a free replacement.

The ⚠ label means: "Do not remove securing screw as otherwise laser beam will escape. For use by service only!"

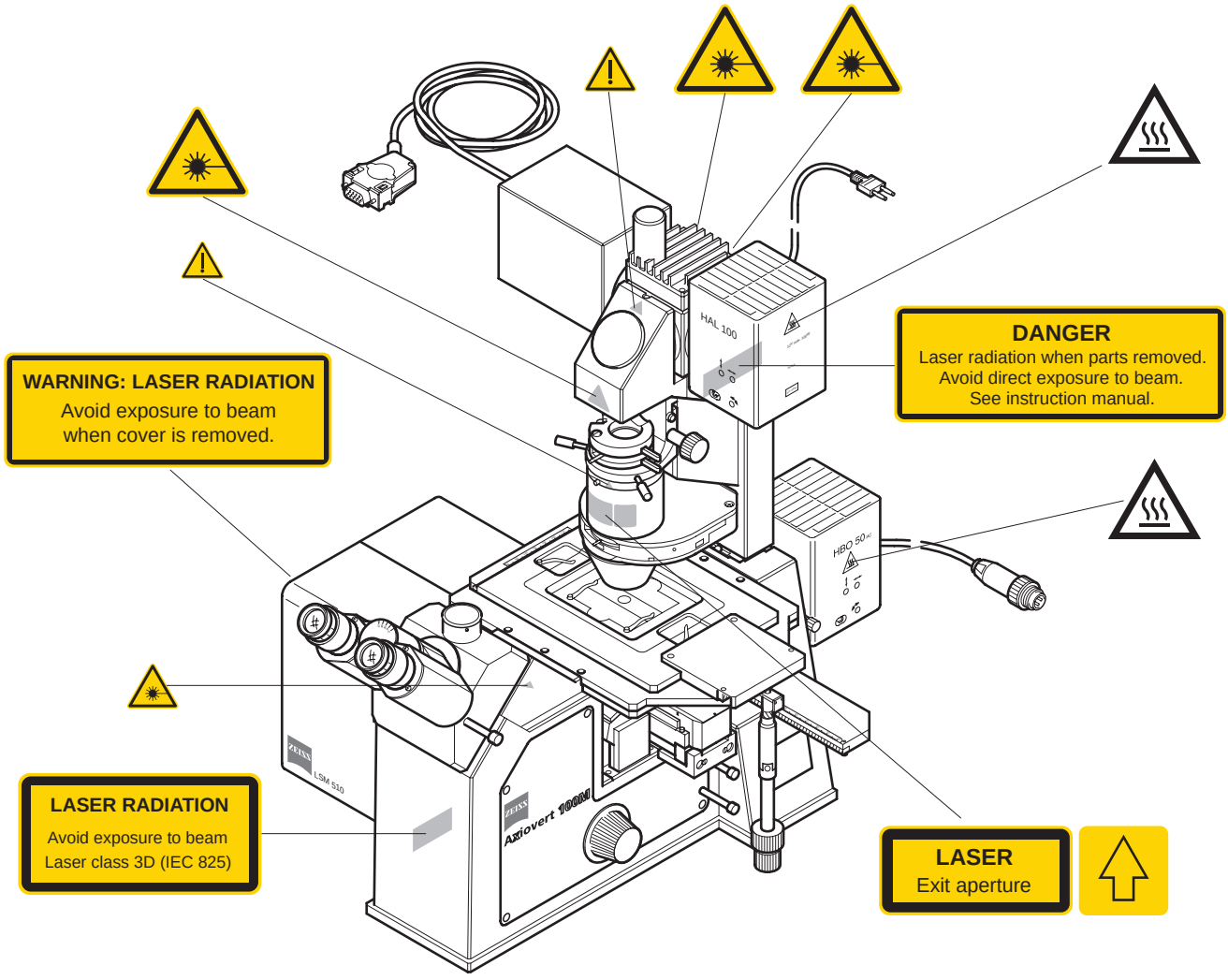


Fig. 1-1 Warning and information labels on the Axiovert 100 M microscope with the LSM 510 scanning module

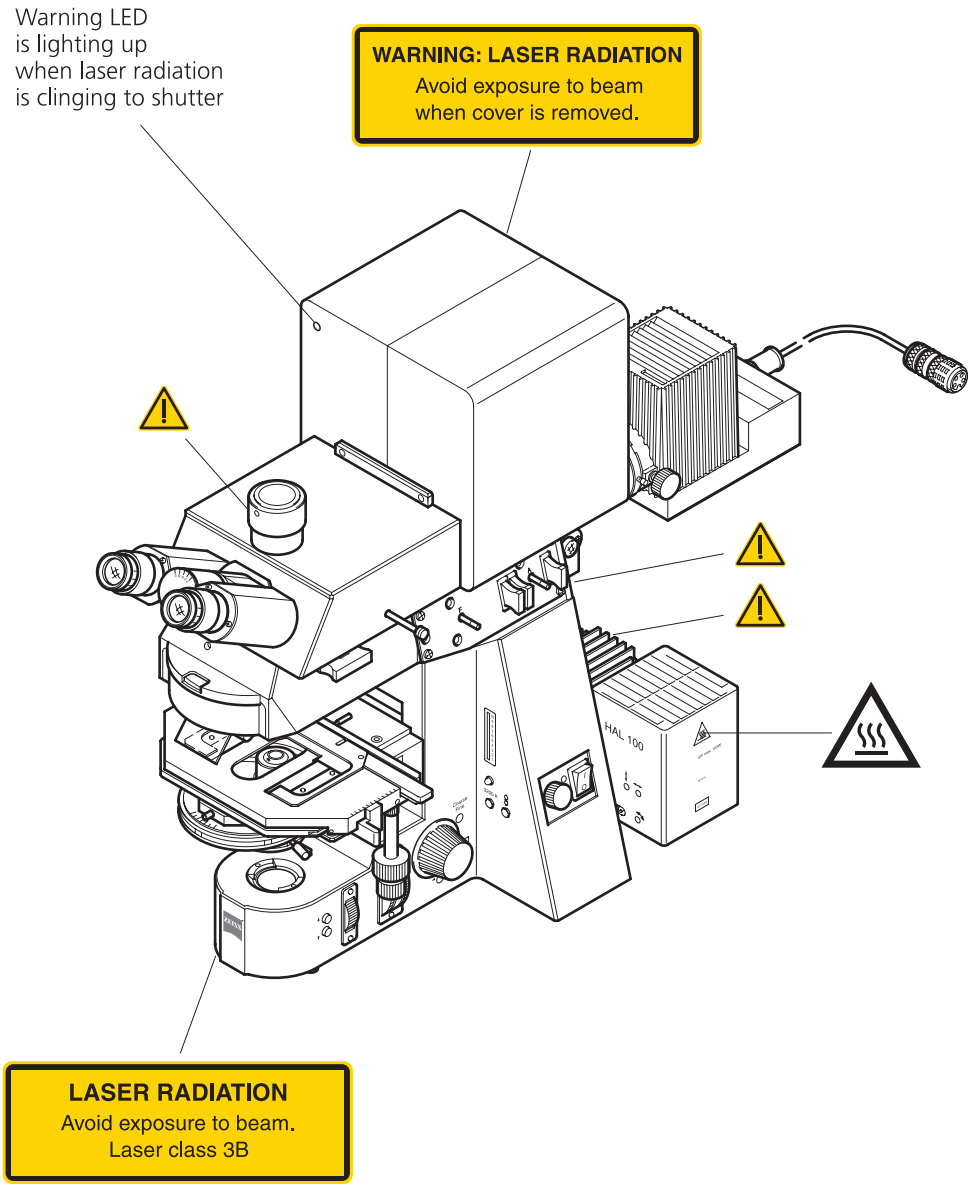


Fig. 1-2 **Warning and information labels on the Axioplan 2 microscope with LSM 510 scanning module**

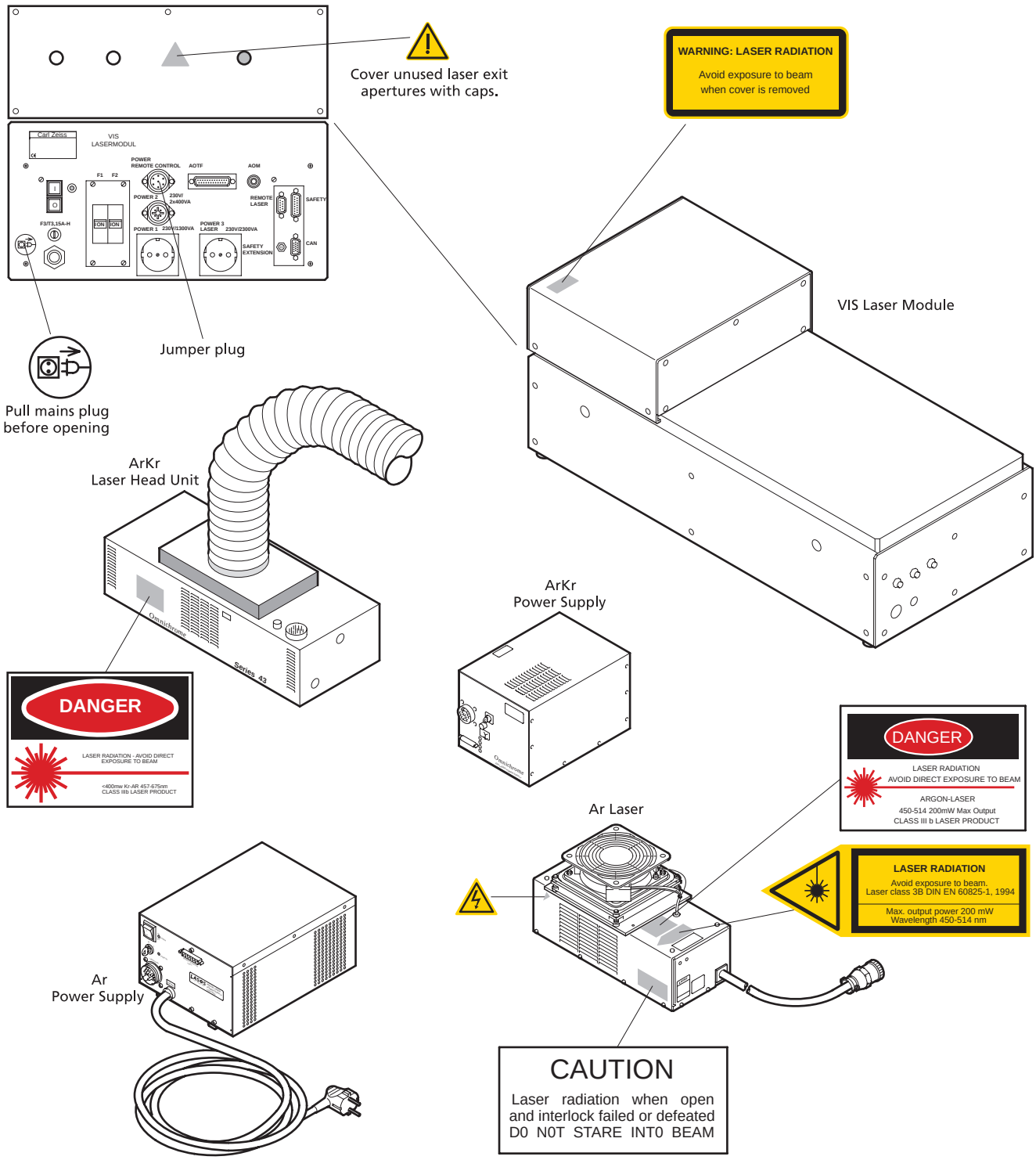


Fig. 1-3 Warning and information labels on laser components (page 1)

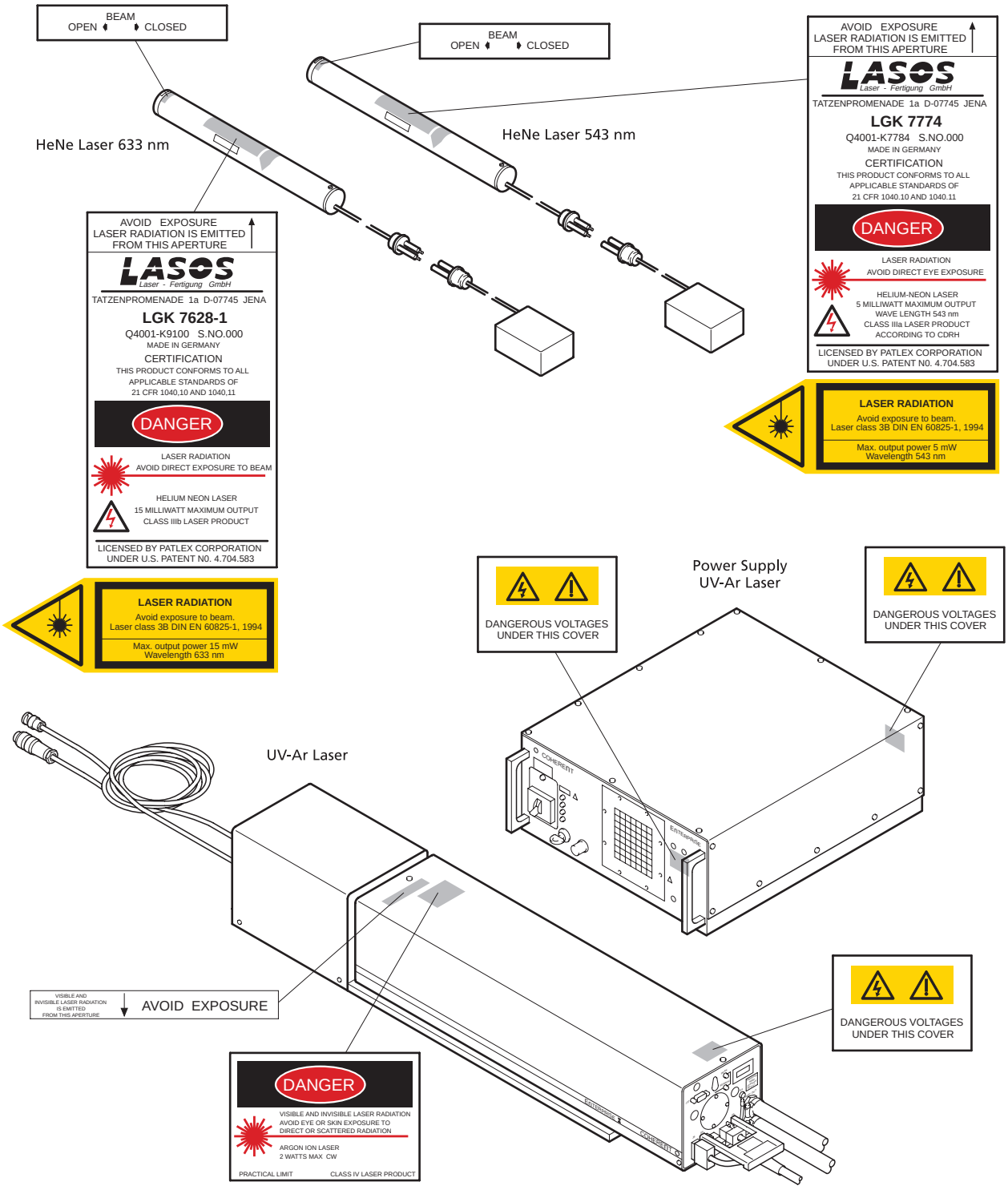


Fig. 1-3 Warning and information labels on laser components (page 2)

CHAPTER 2 LSM 510 - SETUP REQUIREMENTS

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2 LSM 510 - SETUP REQUIREMENTS

2.1 Space Requirements

2.1.1 LSM (one microscope, large system table): 320 cm x 220 cm

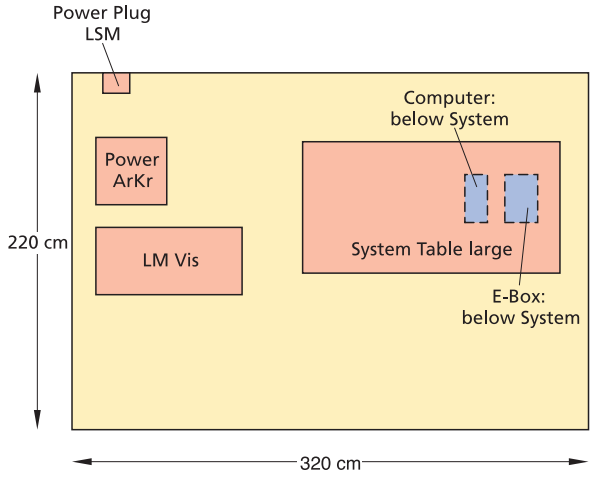


Fig. 2-1

2.1.2 LSM with Ar laser (UV): 340 x 260 cm

☞ We recommend placing the cooling unit of the Ar laser (UV) in a separate room to prevent heat accumulation and vibration. Length of the water hose: 400 cm

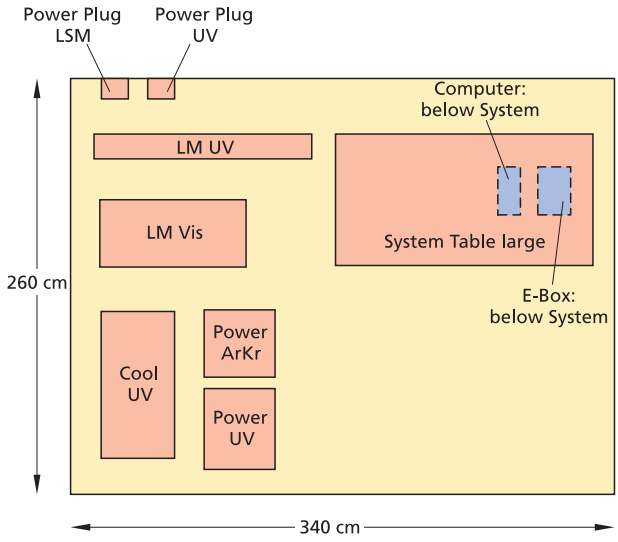


Fig. 2-2

2.1.3 LSM with Ar laser (UV) and two microscopes: 450 x 220 cm

☞ We recommend placing the cooling unit of the Ar laser (UV) in a separate room to prevent heat accumulation and vibration. Length of the water hose: 400 cm.

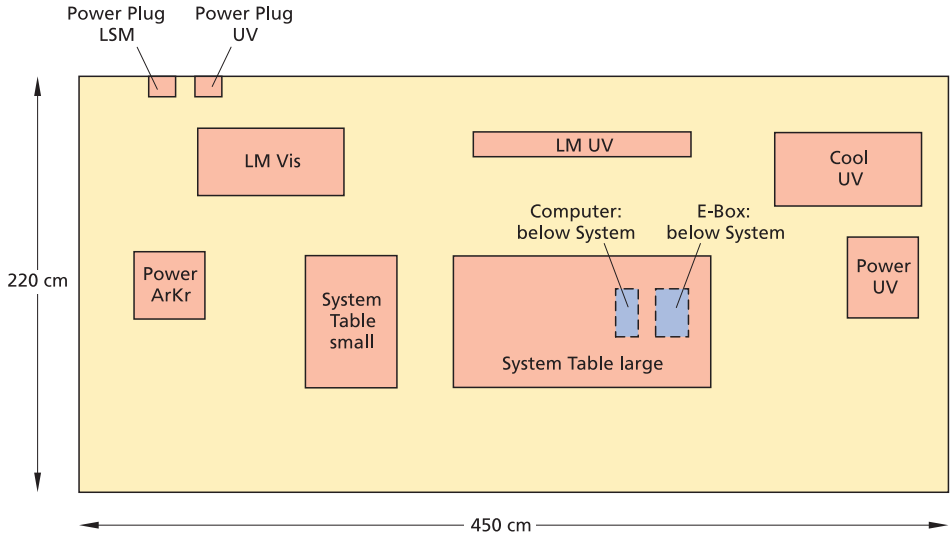


Fig. 2-3

2.2 Power Requirements

☞ The LSM 510 comes with a mains power supply cord and plug, either CEE red (230 V, 16 A, 3 phases), or CEE yellow (115 V, 32 A, 3 phases), and with the matching mains socket outlet.

	Europe	**Japan/USA**
Line voltage	230 V AC: 220...240 V AC (±10 %)	115 V AC: 100...125 V AC (±10 %)
Line frequency	50...60 Hz	50...60 Hz
LSM incl. VIS laser		
– Max. current	2 phases at 16 A Phase 1 = 1.8 kVA max. Phase 2 = 2 kVA max.	2 phases at 25 A Phase 1 = 1.8 kVA max. Phase 2 = 2 kVA max.
– Power consumption	2000 VA per phase	2000 VA per phase
– Power plug	CEE red (230 V, 16 A): 3 phases+N+PE, phases 1 and 2 connected	CEE yellow (115 V, 32 A): 3 phases+N+PE, phases 1 and 2 connected
Argon UV laser		
- Max. current	1 phase at 32 A	1 phase at 63 A
- Power consumption	7400 VA	7400 VA
Class of protection	I	I
Type of protection	IP 20	IP 20
Overvoltage category	II	II
Pollution degree	2	2

☞ If the line voltage in your country is 115 V AC, you need to order an additional 2.5 kW step-up-transformer, part no. 234.366, to be able to run the ArKr laser. Reason: The ArKr laser requires a 220 V input.

Power distribution inside the Laser Module VIS:

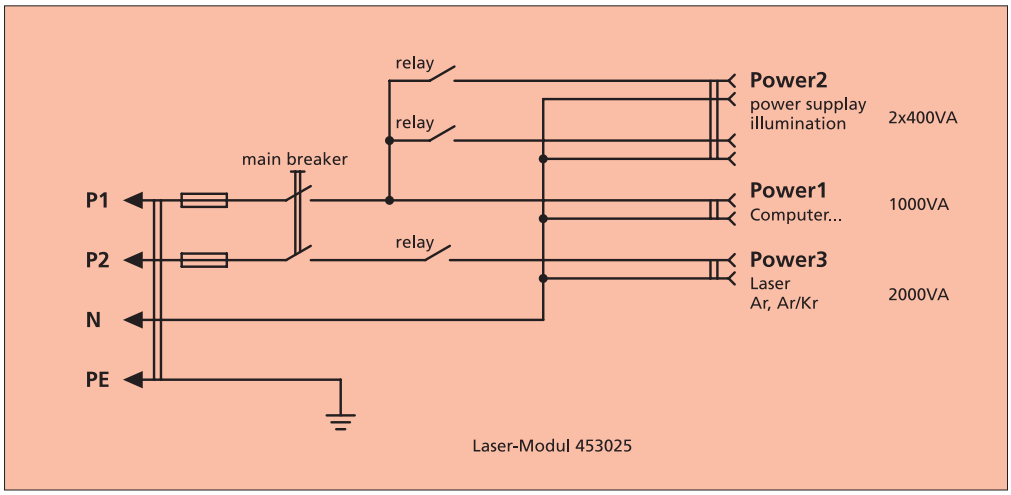


Fig. 2-4

2.2.1 Phase 1 (LSM)

feeds the following units:
- Laser Module
- HeNe 2x
- via Power 1 (5-socket adapter)
  - Computer + monitor
  - Microscope
  - MCU 28
  - Scanning Module
- via Power 2:
  - HAL lamp
  - HBO lamp

2.2.2 Phase 2 (LSM, Power 3)

feeds the following units:

Ar laser	2 kW
or ArKr laser	2 kW

2.2.3 Separate connection:

Ar laser (UV)	7 kW

2.3 Physical Dimensions

	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Large system table	150	80	78	100
Small system table	65	80	78	60
Scanning Module	25	20	25	15
Microscope	50	35	50	20
Laser Module, VIS(ible light)	80	40	50	60
Laser Module, UV	140	20	20	60
Electronics box	50	30	30	10
Power supply for Ar, ArKr	30	30	20	10
Power supply for Ar (UV)	50	50	30	30
Cooling unit for Ar (UV)	80	45	50	30
Water hose for Ar (UV)	400			
Fiber optic cable, VIS(ible)	200			
Fiber optic cable, UV	200			
Cables	250			
SCSI cable	100			

2.4 Dimension of shipment crates

Crate containing	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Large system table	160	85	95	120
Small system table	90	75	80	80
LSM	190	85	120	350
Monitor, computer	120	80	90	80
UV laser unit	125	55	50	100
UV cooling unit	120	60	90	50

2.5 Environmental Requirements

Operation, specified performance	T = 22 °C ±3 °C
Operation, reduced performance	T = 10 °C to 35 °C
Storage, less than 16h	T = -40 °C to 55 °C
Storage, less than 6h	T = -55 °C to 70 °C
Temperature gradient	±3 °C/h
Warm up time	1 h
Relative humidity	< 65 % at 30 °C

2.6 Vibrations

Vibrations under operation conditions (with system table)	**Shipping shock (LSM 5 box)**
5 µm pp at 5 Hz	3 g
10 µm pp at 10 Hz	
10 µm pp at 20 Hz	

2.7 Laser Specifications

2.7.1 Coherent Enterprise 653 II: 352, 364 nm, 80 mW, laser power class 3 B

Line voltage	100...240 V
Line frequency	50...60 Hz
Max. current	1 phase at 32...63 A
Power consumption	7400 VA
With heat exchanger LP5:	
Water flow	8.0 l/min (max 16 l/min)
Water pressure	1.4...4.2 kg/cm²
Water temperature	10...60 °C at 8.0 l/min
Power to water cycle	max. 4500 W
Power from power supply	max. 300 W
Power from LP5	max 6000 W

2.7.2 Uniphase M. 1674 P: 543 nm, 1 mW

Line voltage	100...240 V
Line frequency	50...60 Hz
Power consumption	20 VA

2.7.3 LASOS LGK 7628-1: 633 nm, 5 mW

Line voltage	100...240 V
Line frequency	50...60 Hz
Power consumption	20 VA

2.7.4 LASOS LGK 7812 ML-1/LGN 7812: 458, 488, 514 nm, 25 mW, laser power class 3 B

Line voltage	100...240 V
Line frequency	50...60 Hz
Max. current	1 phases at 25 A
Power consumption	2000 VA
Cooling fan	on top of laser head

2.7.5 Omnichrome 50YB 643/171B: 488, 568 nm, 30 mW, laser power class 3 B

Line voltage	208...240 V
Line frequency	50...60 Hz
Max. current	1 phase at 16 A
Power consumption	2000 VA
Distance to external fan	100 mm
Power from laser head	max. 300 W
Power from power supply	max 6000 W

2.8 Microscopes

	Inverted Axiovert 100 M BP or SP
	Upright Axioplan 2 MOT
	All Zeiss ICS objectives and accessories can be accommodated.
Z motor	DC servomotor, opto-electronically coded
	Least Z interval: 100 nm
HRZ-200	Galvanometer-driven precision focusing stage
	Max. travel 200 µm; resolution 6 nm; accuracy 40 nm
	Allows continuous Z-scan at up to 10 Hz

2.9 Scanning Module

	2 individually driven galvanometric scanners
Scanning speed	Up to 2.6 frames/sec (512 x 512 pixels)
Field resolution	Max. 2048 x 2048 pixels (individually adjustable for each axis)
Field of view	10 x 10 mm² with a 1.25x objective
Zoom	1x ... 8x, continuous control
Channels	Up to 4 channels simultaneously
	4 confocal reflection/fluorescence channels (PMT)
	1 transmitted light channel (PMT)
	1 reference monitor diode
	Cooled PMTs (option, forthcoming)
	Fiber-optic adaptation of external detectors (option, forthcoming)
Dynamic range	12-bit DAC for each detection channel
Pinholes	4 individual variable pinholes (for each confocal channel)
	Computer controlled automatic alignment

2.10 Laser Module VIS

Single-mode polarization preserving fiber

Laser beam attenuation for all lasers by VIS-AOTF

HeNe laser (543 nm, 1 mW)

HeNe laser (633 nm, 5 mW)

Ar laser (458, 488, 514 nm, 25 mW)

Ar laser (488 nm, 15 mW)

ArKr laser (488, 568 nm, 30 mW)

Fuses and automatic circuit breakers

for 230 V:	G-type fuse 5 x 20 mm; slow-blow 3.15 A / H / 250 V, acc. to IEC 127
	2 circuit breakers; C 10 A
for 110 V:	G-type fuse 5 x 20 mm; slow-blow 3.15 A / H / 250 V, acc. to IEC 127
	Circuit breaker; B 25 A
	Circuit breaker; C 25 A
	Circuit breaker; B 16 A
	Circuit breaker; B 10 A

2.11 Laser Module UV

Single-mode polarization preserving fiber

Laser beam attenuation for all lasers by UV-AOTF

Ar laser (351, 364 nm, 80 mW)

CHAPTER 3 INTRODUCTION TO LASER SCANNING MICROSCOPY

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3 INTRODUCTION TO LASER SCANNING MICROSCOPY

3.1 Principle of Laser Scanning Microscopy

To yield information on their inner structure by conventional transmitted-light microscopy, specimens have to be very thin and translucent; otherwise image definition will be poor. In many cases it is a problem to satisfy these requirements.

The essential considerations have led to trailblazing changes in conventional microscopy and supplied a successful solution to the above problem.

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- The light reflected, or the fluorescence light produced, at the focus of the high-NA objective is projected onto a variable pinhole diaphragm by the same objective and a tube lens. The focus inside the specimen and the pinhole are situated at optically conjugate points **(confocal imaging).** The decisive advantage of this arrangement is the fact that essentially no other light than that coming from the object plane of interest can pass the narrow pinhole and be registered by a detector. Unwanted light coming from other specimen areas is focused outside the pinhole, which passes only a small fraction of it. The smaller the pinhole, the less stray light or fluorescence from out-of-focus areas will get on the detector. The image point thus generated is largely free from blur caused by unwanted light.

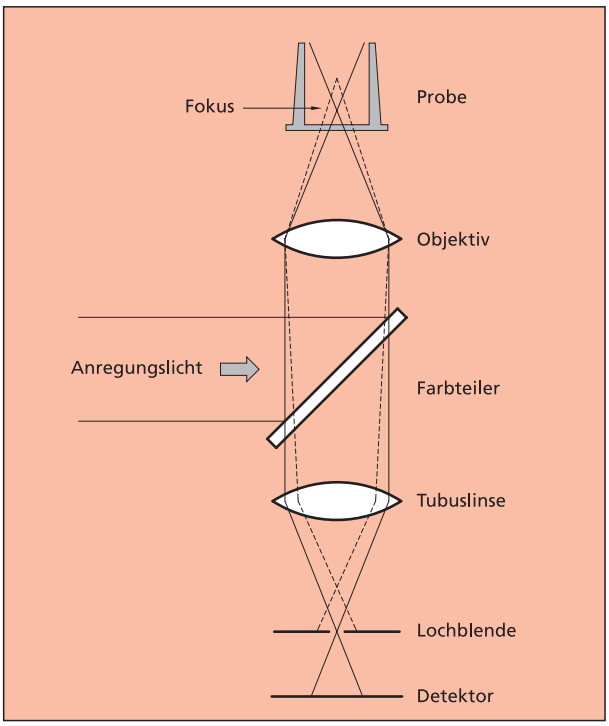


Fig 3-1 Principle of confocal imaging

- In order to obtain an image of the selected object plane as a whole, it is necessary to scan the object plane in a point-by-point, line-by-line raster by means of an XY light deflection system. The detectors - as a rule, photomultipliers - convert the optical information into electric signals. This allows the image of any object plane to be generated and stored within less than a second. By a defined focusing (Z axis) movement it is possible to look at any object plane of interest. By scanning a succession of object planes in a specimen, a stack of slice images can be produced.

This way, the LSM technique in conjunction with ICS optics (Infinity Color-Corrected System) has brought decisive improvements over conventional microscopy in terms of resolving power and confocal depth contrast:
Object features in the order of 0.2 µm can be resolved, and height differences of less than 0.1 µm made visible, without the use of interference methods.

3.2 Three-Dimensional Presentations of LSM Image Stacks

One of the advantages of the LSM technique is that it can present structures in three dimensions. This opens up many ways to process images. Outlined below are some of the possible methods to extract spatial information from stacks of slice images.

- **Gallery**
  The simplest presentation of 3D information is a gallery showing the individual slice images (sections) of a stack arranged side by side, with each slice apart from the next by a defined, selectable interval on the Z axis.
- **Virtually infinite depth of focus**
  The entire set of data can be imaged as a single projection. The computer establishes an image composed of all in-focus optical sections. The image produced by this so-called composite method has a virtually infinite depth of focus, since the result is made up of information from in-focus planes only.
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  A sequence of projections is computed, with the specimen being apparently rotated by a certain angle from image to image, for example by a full turn about an axis. If such a sequence is displayed on the monitor screen in rapid succession, the visual effect is that of a rotating three-dimensional object.
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  Each level, i.e. each slice is assigned a different color. For direct evaluation, a color scale is shown, indicating the actual height above the bottom slice.

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  This computation produces a triplet of mutually perpendicular sectional images.
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  A section through the stack is made along an oblique plane defined by the selection of five coordinates, i.e. X, Y, Z, angle of rotation, and angle of tilt.
- **Topography** (forthcoming)
  A computing program for surface topography presentations (as required in materials research) is available.
- **Kinetics** (forthcoming)
  With a special software, kinetic processes can be tracked, which is especially of interest to physiology.

3.3 Optical Diagram of the LSM 510 (Schematic)

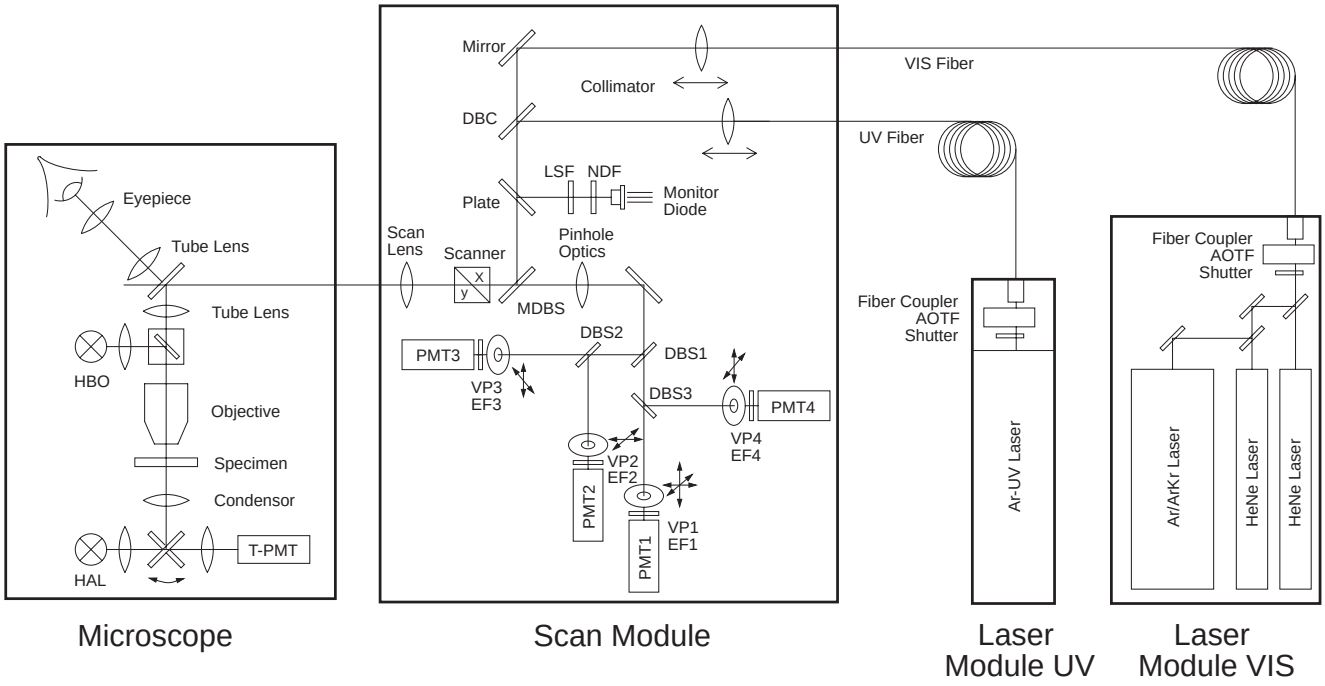


Fig. 3-2 Optical path, schematic (4-channel configuration)

AOTF	Acousto Optical Tunable Filter
DBC	Dichroic Beam Combiner
DBS	Dichroic Beam Splitter
EF	Emission Filter
HAL	Halogen Lamp
HBO	Mercury Vapor Short-Arc Lamp
LSF	Line Selection Filter
MDBS	Main Dichroic Beam Splitter
NDF	Neutral Density Filter
VP	Variable Pinhole
PMT	Photomultiplier
T-PMT	Transmission-Photomultiplier

The diagram above is a schematic representation of the LSM system.

Laser light is focused onto the specimen through an objective in a diffraction-limited mode. Light emitted at the focal plane and at planes below and above it is directed via an XY scanner onto a main dichroic beam splitter (MDBS), which separates the emissions from the excitation light. The fluorescences are separated from each other by a series of dichroic beam splitters (DBS1 ... maximally DBS4) and directed to individual photomultipliers (PMT1 ... maximally PMT4).

3.4 Performance Features of the LSM 510

3.4.1 Optical and mechanical aspects

The highly integrated system design makes for the shortest possible optical paths, top-grade optical precision and high stability. The compact scanning module can be fitted to an inverted (Axiovert 100 M BP or SP) or upright (Axioplan 2 MOT) microscope in less than three minutes. On the Axiovert, the scanning module may be mounted either to the base port directly below the microscope or to the side port.

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Acousto-optical tunable filters (AOTF) adjust the necessary brightness for all desired laser lines within microseconds.

A monitor diode permanently registers the laser output; it can be used for the on-line checking of the intensity of the exciting light. This check is also possible selectively for the different wavelengths if a line selection filter is inserted.

The four simultaneous image acquisition channels, usable for reflection or fluorescence, and an additional transmitted-light channel are ideal for the investigation of multiple fluorescence specimens. Separately in each of the four channels, the diameters of the pinholes and their XY positions can be optimized, and the desired emission filter placed into the beam path, by servo-motor control. In the case of pinhole VP1, this adjustment also includes positioning along Z. In the simultaneous registration of multiple fluorescences, identical optical sections can be obtained in each confocal channel. This is of importance, e.g., with the FISH method (fluorescence in-situ hybridization) used for genome analysis in cytogenetic studies.

The microscope's transmitted-light channel is equipped with a photomultiplier, too. It is therefore possible to superimpose a multiple fluorescence image on a brightfield, differential interference or phase image.

A fiber-optic cable connection to external special detectors, such as cooled PMTs or spectrometers, is under development.

In addition to the emission filters for all standard and special applications, available in motor-controlled filter wheels, the user can easily install his own emission filters in two of the channels.

The high-NA C-APOCHROMAT objectives specially developed for the LSM technique reach the physical limit in resolving power, and can be used throughout the 350...700 nm spectral range with the same high quality, producing brilliant images.

A two-mirror scanner system, controlled by a digital signal processor (DSP), offers several advantages. The large deflection angle of the scanning mirrors allows a wide area to be scanned. With a 1.25x objective, the object area scanned is 10 x 10 mm².

The scanning field size can be freely selected between 2 x 2 and 2048 x 2048 pixels.

It is possible to rotate the XY scanning field through 360° and carry out XY scans without having to rotate the specimen itself under laser radiation load.

Selection of the specimen detail of interest for zooming is fast and convenient, and the zoomed image is automatically centered. This saves the job of specimen centration with the microscope stage.
Using a bi-directional scanning facility (forthcoming) will double the scanning rate to 2.6 frames/sec (at 512 x 512 pixels); if two different lasing wavelengths are used for the two scanning directions (wavelength 1 for left-to-right, and wavelength 2 for right-to-left scanning), two fluorochrome dyes can be viewed and documented in a quasi-simultaneous mode. This will absolutely prevent "bleeding".

3.4.2 Microscope equipment of the LSM 510 system

The LSM 510 system is equipped either with the Axiovert 100 M BP or SP microscope which is founded on the Axiovert 100/135 microscope serie, or with the Axioplan 2 MOT microscope.

Referring to the delivered operating manual "Axiovert 100, Axiovert 135 and 135 M Transmitted and fluorescent reflected light" only differences to this manual will be explained.

(1) Stand

a) The motorized objective nosepiece 5x H DIC is firmly fixed to the stand, where no operating elements can be found for the nosepiece. Operation will be done LSM 5 software controlled. The "Restriction of revolver hight to protect the objectives when changing the objectives motorized" is inactivated. The nosepiece will be moved down automatically before each motorized objective change.

b) The reflector mount is motorized and provided with the LSM 5 Axiovert reflector slider (451333). The reflector slider has 4 positions: One transmitting light position, which is identically the LSM position and three further positions for fluorescence filter sets. If you want to use more than three conventional fluorescence filter sets it is advisable to use further reflector sliders (451333). When changing the reflector slider you have to look that the slider will click into place otherwise the image area will be cutted.

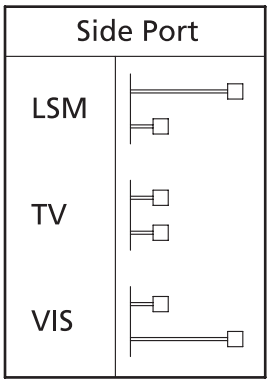
c) The stand has a motorized focusing drive. Switching between fine and coarse drive can be done by push button on the left beside the focusing drive. Sensitivity of the focusing drive is adjusted to the delivered objectives by manufacturer. If you want to use other objectives, sensitivity and parfocality can be adjusted with the CLM program (configuration and light manager). After changing an objective the focusing drive will be located in fine focusing mode.

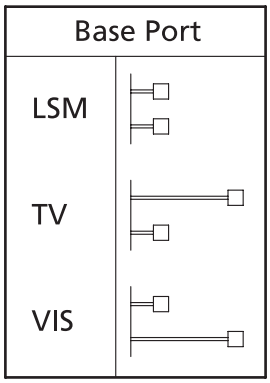
d) The stand featuring an integrated power supply for the internal motors and stand electronics. The power supply can be switched-on at rear side of the stand. External power supply units will be used for the mains of halogen lamp or mercury vapour short arc lamp.

e) The analyzer slider for conventional DIC methods will be operated from the right side and is located just below the nosepiece.
When the rod is pushed in, the analyzer is located in the beam path. In LSM-mode the analyzer must **not** be located in the beam path, analyzer rod must be pulled out.

f) The stands dispose of two additional ports, a side port and a base port respectively.
One of these ports is equipped with the LSM 5 special interface, the other one with the TV interface. The LSM 5 scanning module can be mounted to the special interface port. Different camera systems can be adapted to the TV interface using the TV adapters 452982/83/92/94/95/97/98.
The light reaches the ports over full mirrors. Therefore all light is available at side port, base port or for conventional microscopy.
Bringing the beam path to the desired direction you must use the two rods on the right side of the stand.
The upper rod is designed to the side port. When the rod is pulled out, all the light is directed to the side port.
The lower rod is designed for the base port. When the rod is pushed in, all the light is directed to the base port, however the upper rod must be pushed in too.
To direct light to the tube, the upper rod must be pushed in, the lower rod must be pulled out.

Logic scheme of the rods:





(2) Specimen stages

a) Mechanical stage 000000-1017-918
The stage must be mounted with the coaxial drive on the right side of the stand.

b) Scanning stage 000000-1017-917
The scanning stage can only be used, when the LSM 5 scanning module is mounted to the base port.

(3) Transmitted-light illumination

a) The illuminator support contains a security circuit, which activates a shutter preventing laser light from reaching the stand when the support is moved to back. A complementary shutter built-in the stand prevents laser light from reaching the eye pieces during scanning mode.

b) The illuminator support is equipped with a rotatable polarizer. The Axiovert description contains the adjustment for DIC mode during conventional observation.
For scanning transmitted light DIC mode the polarizer in the transmitted light support works like an analyzer and must be adjusted in such a manner, that direct laser light will be blocked.
The conventional analyzer slider in the stand is not allowed be located in the beam path because of the laser light already is polarized.

c) On the illuminator support as an option there is mounted a LSM 5 software controlled switching mirror fully motorized. Alternatively the light is directed to the LSM 5 T-light detector or enables conventional transmitted-light observation.

d) The focusing screen for conventional transmitted-light is located in a support in front of the halogen lamp housing.

e) Further information to halogen lamp and condensers you will find in the Axiovert operating manual.

(4) Reflected light fluorescence

All Axiovert fluorescence accessories exceptional the reflector slider can be used.
Further information you will find in the Axiovert operation manual.

(5) Imaging optics

Optovar sliders are not usable.
The analyzer for conventional DIC mode will be operated from the right side and is located just below the nosepiece.
Use of sliders with auxiliary objects (473704/14) is not possible.

(6) Photo equipment

The stand doesn´t have an integrated SLR-port, but microscope cameras, as described in the Axiovert operation manual, can be used.

(7) TV adaption

The TV port aside and the tubes can be used as described in the Axiovert operation manual.

The TV interface side port or base port can only be used with TV adapters 44.

3.4.3 Computer hardware and software

The LSM 510 is controlled through a standard high-end Pentium PC. Linking with the electronic control system is via an ultrafast SCSi interface. The PC comes with the 32-bit WINDOWS NT 4.0 operating system.

The instrument is fully motorized, permitting fast change-over between methods as well as automatic operation. Parameters once set or complex examination sequences once established can be saved and reproduced; this way, complete application programs can be loaded and executed by pushbutton control.

The software of the LSM 510 has two levels. On the simple operator interface level, a result will be achieved after a few prompts; graphical prompting of the user in conjunction with automatic setting of many parameters is an ideal tool for daily routine jobs. The expert level offers perfect facilities for individual settings of functions and parameters.

Conversion of the light signals into a digital image is effected by means of four 12-bit A/D converters, each of which can generate 4096 brightness levels.

The software provides a enormously wide range of image processing functions, including all standard 2D/3D (stereo, projection) functions same as sophisticated 3D reconstruction capabilities (surface and alpha rendering), digital processing of voxels and 3D measurement functions (surface areas, volumes).

As all files and images are recorded in MS Access databases, elegant image database editing is just as easy as transferring the records to other programs.

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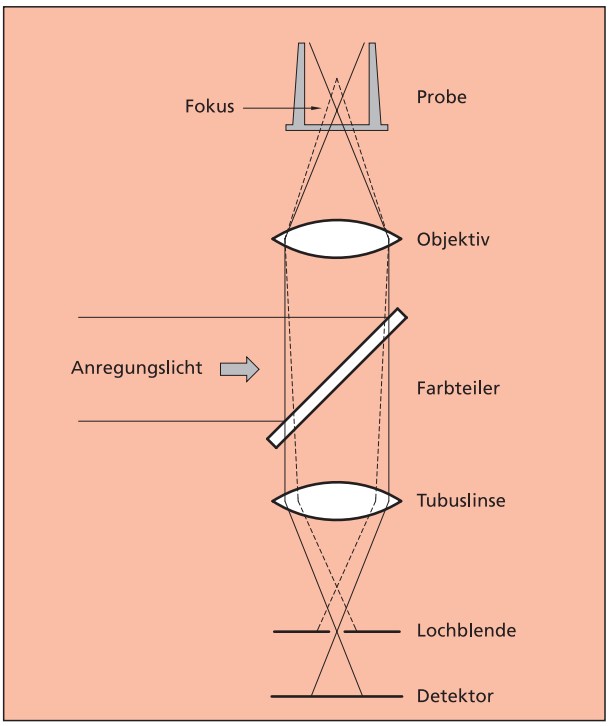


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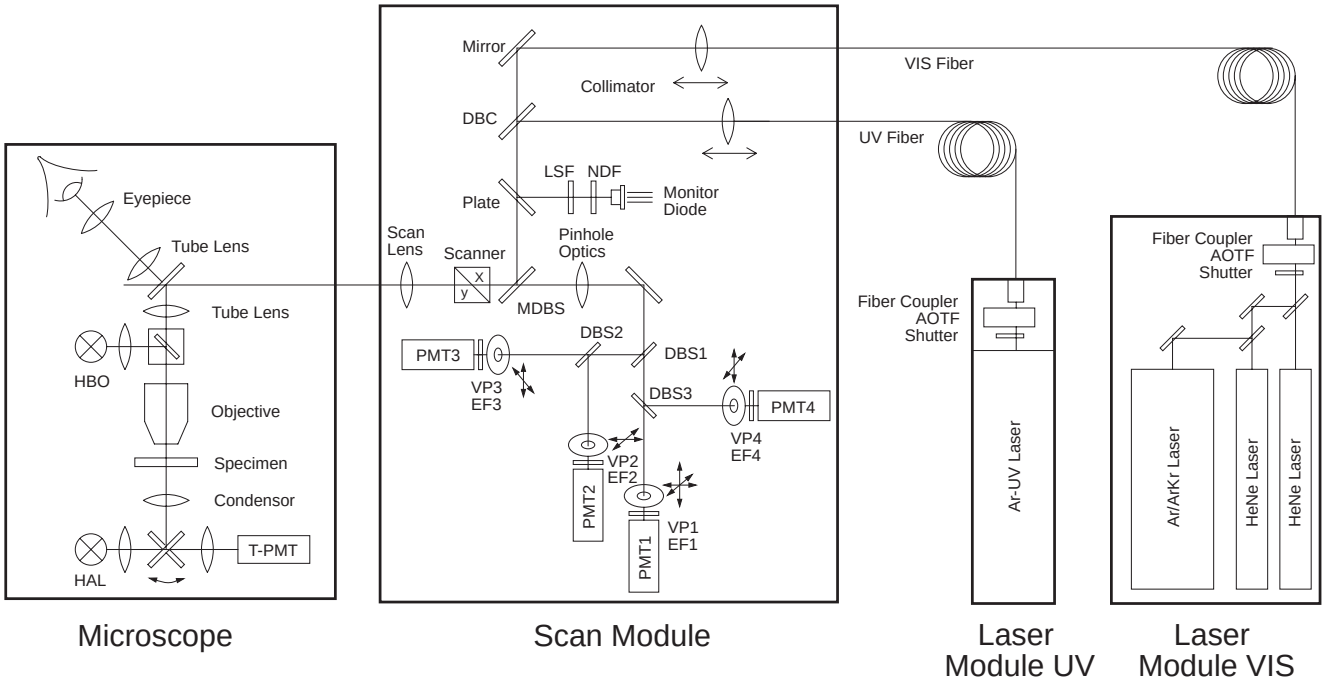


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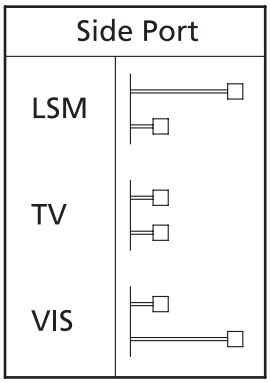
c) The stand has a motorized focusing drive. Switching between fine and coarse drive can be done by push button on the left beside the focusing drive. Sensitivity of the focusing drive is adjusted to the delivered objectives by manufacturer. If you want to use other objectives, sensitivity and parfocality can be adjusted with the CLM program (configuration and light manager). After changing an objective the focusing drive will be located in fine focusing mode.

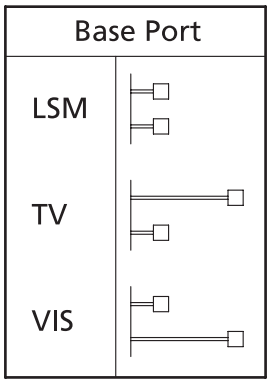
d) The stand featuring an integrated power supply for the internal motors and stand electronics. The power supply can be switched-on at rear side of the stand. External power supply units will be used for the mains of halogen lamp or mercury vapour short arc lamp.

e) The analyzer slider for conventional DIC methods will be operated from the right side and is located just below the nosepiece.
When the rod is pushed in, the analyzer is located in the beam path. In LSM-mode the analyzer must **not** be located in the beam path, analyzer rod must be pulled out.

f) The stands dispose of two additional ports, a side port and a base port respectively.
One of these ports is equipped with the LSM 5 special interface, the other one with the TV interface. The LSM 5 scanning module can be mounted to the special interface port. Different camera systems can be adapted to the TV interface using the TV adapters 452982/83/92/94/95/97/98.
The light reaches the ports over full mirrors. Therefore all light is available at side port, base port or for conventional microscopy.
Bringing the beam path to the desired direction you must use the two rods on the right side of the stand.
The upper rod is designed to the side port. When the rod is pulled out, all the light is directed to the side port.
The lower rod is designed for the base port. When the rod is pushed in, all the light is directed to the base port, however the upper rod must be pushed in too.
To direct light to the tube, the upper rod must be pushed in, the lower rod must be pulled out.

Logic scheme of the rods:





(2) Specimen stages

a) Mechanical stage 000000-1017-918
The stage must be mounted with the coaxial drive on the right side of the stand.

b) Scanning stage 000000-1017-917
The scanning stage can only be used, when the LSM 5 scanning module is mounted to the base port.

(3) Transmitted-light illumination

a) The illuminator support contains a security circuit, which activates a shutter preventing laser light from reaching the stand when the support is moved to back. A complementary shutter built-in the stand prevents laser light from reaching the eye pieces during scanning mode.

b) The illuminator support is equipped with a rotatable polarizer. The Axiovert description contains the adjustment for DIC mode during conventional observation.
For scanning transmitted light DIC mode the polarizer in the transmitted light support works like an analyzer and must be adjusted in such a manner, that direct laser light will be blocked.
The conventional analyzer slider in the stand is not allowed be located in the beam path because of the laser light already is polarized.

c) On the illuminator support as an option there is mounted a LSM 5 software controlled switching mirror fully motorized. Alternatively the light is directed to the LSM 5 T-light detector or enables conventional transmitted-light observation.

d) The focusing screen for conventional transmitted-light is located in a support in front of the halogen lamp housing.

e) Further information to halogen lamp and condensers you will find in the Axiovert operating manual.

(4) Reflected light fluorescence

All Axiovert fluorescence accessories exceptional the reflector slider can be used.
Further information you will find in the Axiovert operation manual.

(5) Imaging optics

Optovar sliders are not usable.
The analyzer for conventional DIC mode will be operated from the right side and is located just below the nosepiece.
Use of sliders with auxiliary objects (473704/14) is not possible.

(6) Photo equipment

The stand doesn´t have an integrated SLR-port, but microscope cameras, as described in the Axiovert operation manual, can be used.

(7) TV adaption

The TV port aside and the tubes can be used as described in the Axiovert operation manual.

The TV interface side port or base port can only be used with TV adapters 44.

3.4.3 Computer hardware and software

The LSM 510 is controlled through a standard high-end Pentium PC. Linking with the electronic control system is via an ultrafast SCSi interface. The PC comes with the 32-bit WINDOWS NT 4.0 operating system.

The instrument is fully motorized, permitting fast change-over between methods as well as automatic operation. Parameters once set or complex examination sequences once established can be saved and reproduced; this way, complete application programs can be loaded and executed by pushbutton control.

The software of the LSM 510 has two levels. On the simple operator interface level, a result will be achieved after a few prompts; graphical prompting of the user in conjunction with automatic setting of many parameters is an ideal tool for daily routine jobs. The expert level offers perfect facilities for individual settings of functions and parameters.

Conversion of the light signals into a digital image is effected by means of four 12-bit A/D converters, each of which can generate 4096 brightness levels.

The software provides a enormously wide range of image processing functions, including all standard 2D/3D (stereo, projection) functions same as sophisticated 3D reconstruction capabilities (surface and alpha rendering), digital processing of voxels and 3D measurement functions (surface areas, volumes).

As all files and images are recorded in MS Access databases, elegant image database editing is just as easy as transferring the records to other programs.

CHAPTER 4 OPERATION

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4 OPERATION

4.1 General

This section describes the operation of the LSM 510 Laser Scanning Microscope exemplified by typical applications in conjunction with the LSM 510 Software and its graphic user environment.

When starting up and operating the microscope system, mind the operating instruction manuals for the Axioplan 2 and Axiovert 100 M microscopes:

- B 40-042 e Axioplan 2, Operating manual
- B 42-513 e Axiovert 100, 135 and 135M, Operating manual

4.2 Software

The LSM 510 Software, Version 2.01, controls the microscope, the scanning and laser modules, tools (objectives, filters, CLM 32 ...) and the image acquisition process, and presents and analyzes the image data. It is based on the network-capable graphic 32-bit Microsoft ® WINDOWS NT 4.0 operating system.

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☞ The installation of the software for the LSM 510 and the basic settings of the equipment components are **exclusively** carried out by Carl Zeiss service staff. This job includes the creation of a customized software configuration in line with the specific hardware components of the customer's microscope system.

A description of how to use the graphic user interface of the WINDOWS NT 4.0 operating system and the LSM program can be found in the Annex.

4.2.1 Boot WINDOWS NT

☞ Drive "A" must not contain a diskette.

Normally, the LSM system is turned on with the REMOTE CONTROL switch. If this switch is not used, turn the system on with the "I" button on the laser module; in addition, the jumper plug supplied must be plugged at the POWER REMOTE CONTROL terminal.

- Turn the REMOTE CONTROL main switch to the "ON" position.
  - Computer boots up.
  - Computer hardware system test runs.

The monitor shows a dialog box for selecting the operating system version.

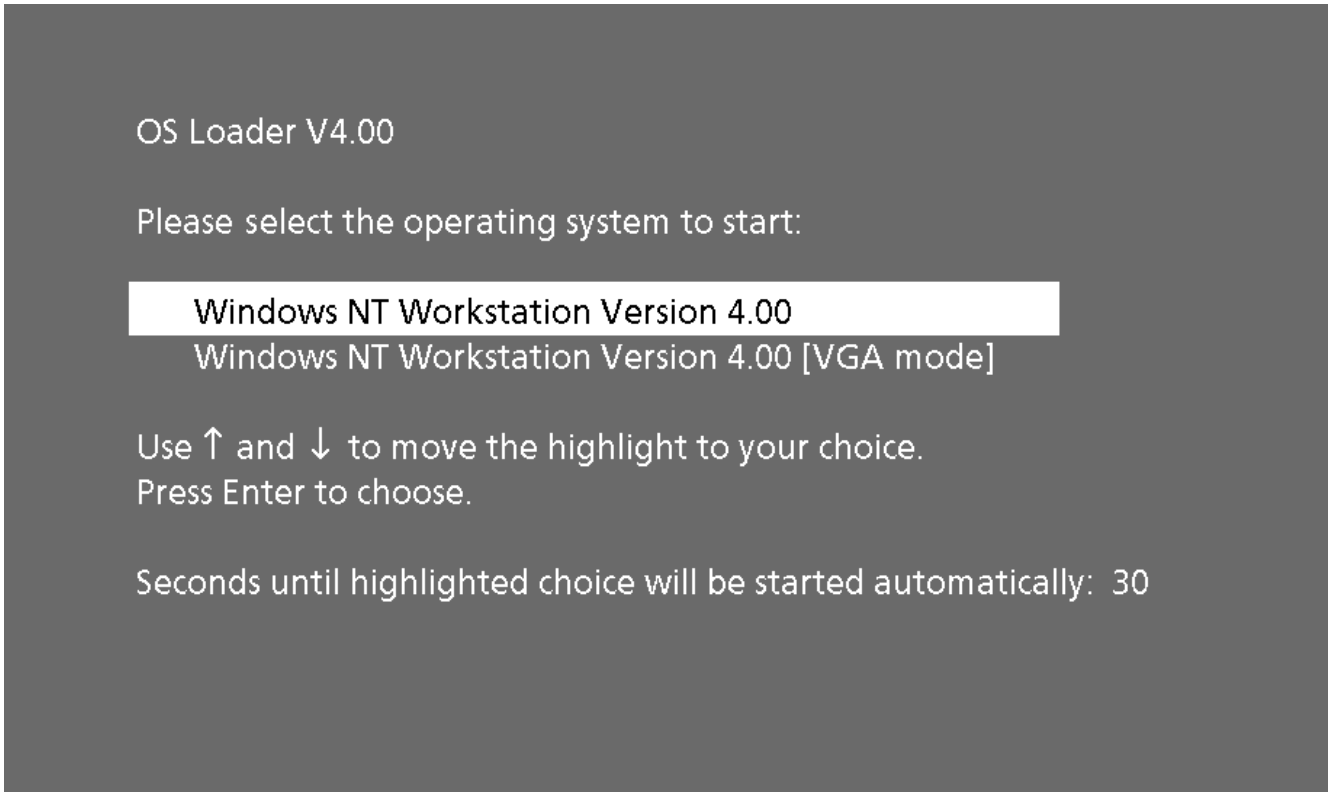

OS Loader V4.00
Please select the operating system to start:
Windows NT Workstation Version 4.00
Windows NT Workstation Version 4.00 [VGA mode]
Use ↑ and ↓ to move the highlight to your choice.
Press Enter to choose.
Seconds until highlighted choice will be started automatically: 30

Fig. 4-1

– WINDOWS NT operating system is being loaded.

– The "Begin Logon" WINDOWS dialog box appears on the monitor.



Fig. 4-2

4.2.2 Log on to WINDOWS NT

- Press the three keys <Ctrl>, <Alt> and <Del> at the same time.
  – The **Logon Information** dialog box appears on the monitor, permitting you to log on to operating system WINDOWS NT 4.0.

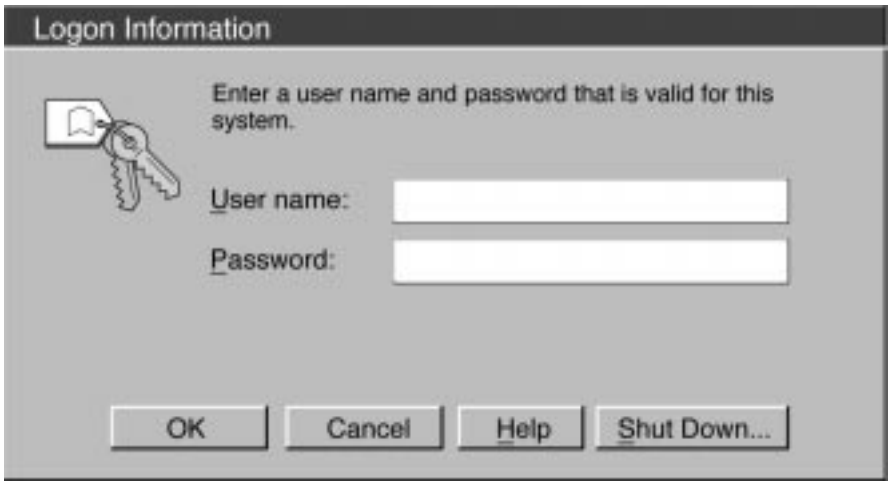


Fig. 4-3

- Enter the declared user name into the **User name** text box.
- Enter your password into the **Password** text box.

- After you have made the two entries, confirm them by clicking on the <OK> button or hitting the <Enter> key.
  - The WINDOWS NT operating system desktop appears on the monitor, showing a number of icons.

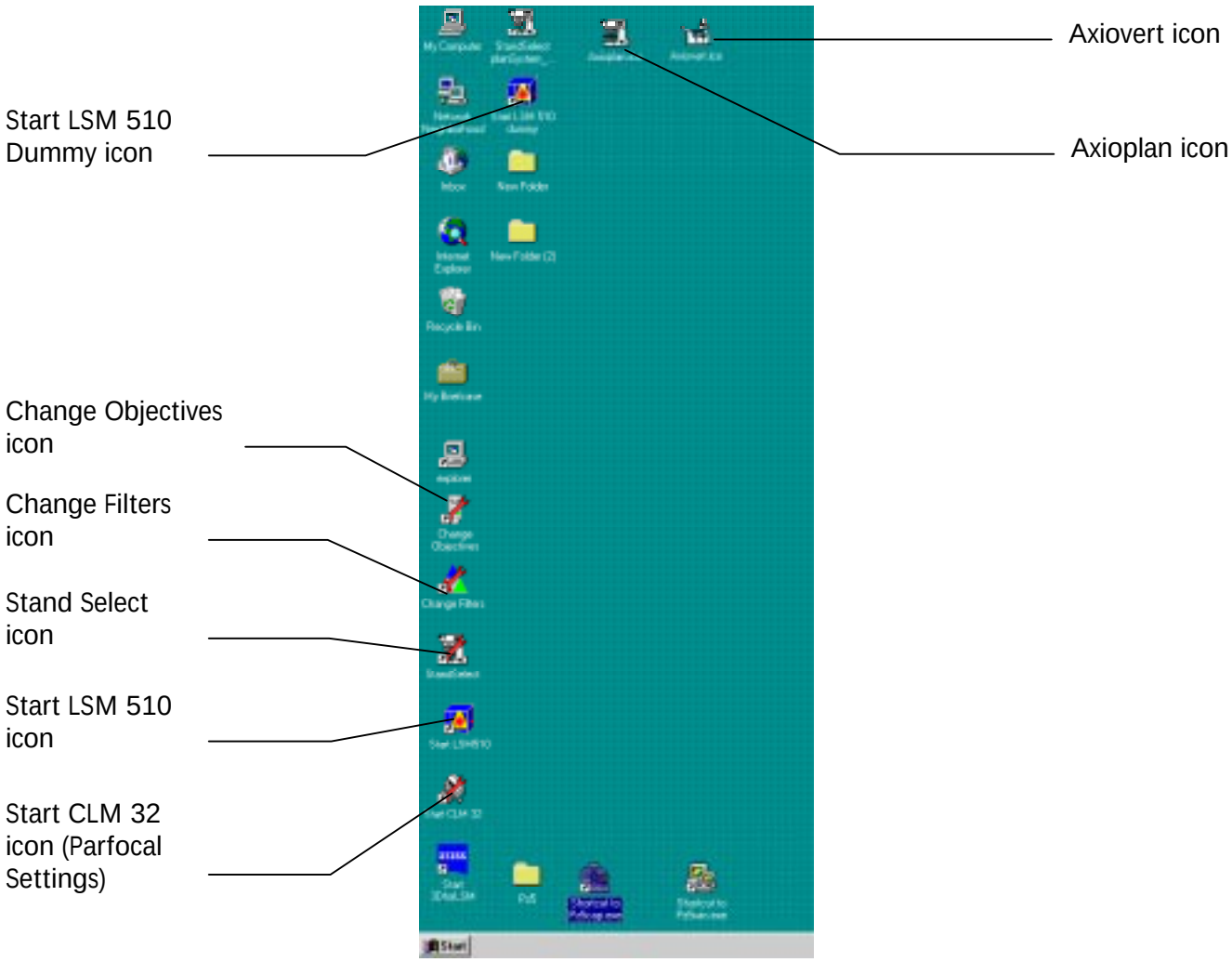


Fig. 4-4

☞ **Start LSM 510 dummy:**
A program that starts the LSM in only dummy mode. Useful in case of working with stored images with a separate workstation or when demonstrating the system without microscope, scan module, electronic box and laser module.
Also a dongle is necessary for operation of the dummy mode.

4.3 Quick start

4.3.1 Starting the LSM Program

(1) From the Windows NT operating system desktop double click on **Start LSM 510** icon. The "LSM 510 Switchboard" menu appears on the screen.



Fig. 4-5

(2) From the LSM 510 Switchboard menu, click on the <Scan New Images> button and <Start Expert Mode> button.
The LSM will go through a CP initialization and open a toolbar labeled LSM 510 expert mode. This tool bar **LSM 510 - Expert Mode** appears on the screen.

4.3.2 Creating a data base for image storage

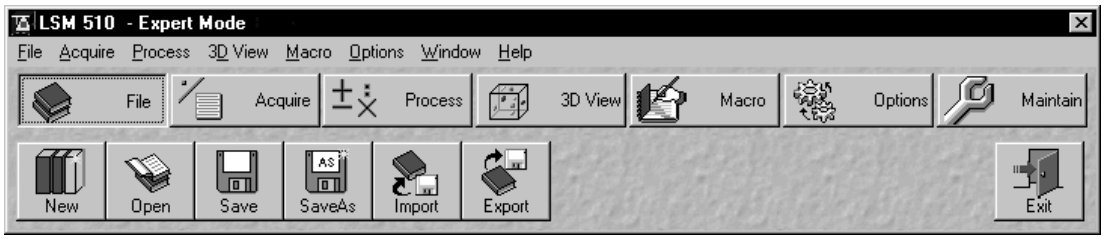


Fig. 4-6

(1) Click on the <File> button from LSM 510 tool bar.

(2) Click on the <New> button.
This will allow you to create a new data base to store your images, experimental setup and comments from your confocal session. If you have previously created a data base, click on the <Open> button instead of <New> button.

(3) Type in a data base name in the **File name** field.
The name can consist of as many characters as you like. Before clicking the create button in the "Create New Database" window, set the location in which the data base will be created by selecting the drive in the **Create in** field, and double-click on the required folder icon from the list displayed.

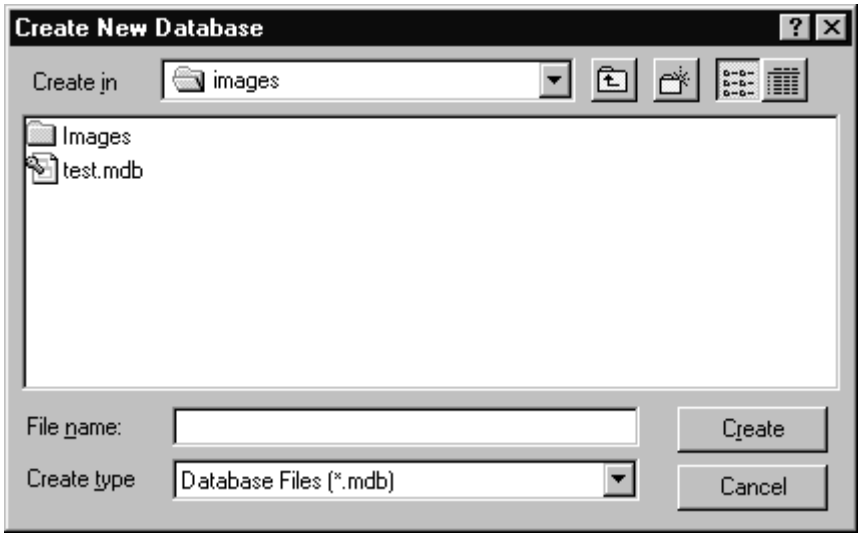


Fig. 4-7

(4) Click on the <Create> button. All images which are saved during your confocal session will be automatically saved in this data base.

4.3.3 Turning the Lasers On

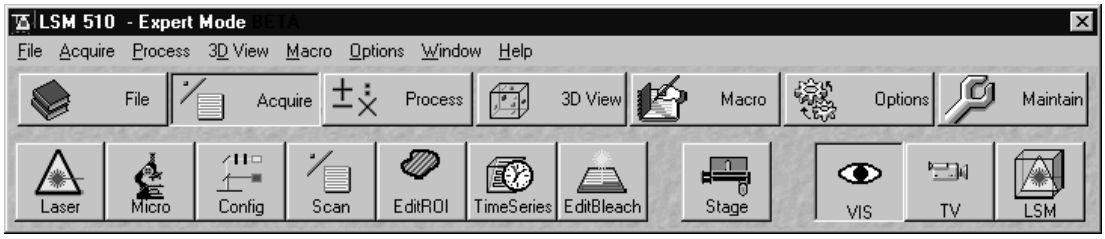


Fig. 4-8

(1) Click on the <Acquire> button from the LSM 510 tool bar.

(2) Click on the <Laser> button.

(3) You will see a **Laser-Control** menu with a list of available lasers. Using the mouse, click on the laser(s) which has the appropriate wavelength to excite the dyes labeling your specimen.

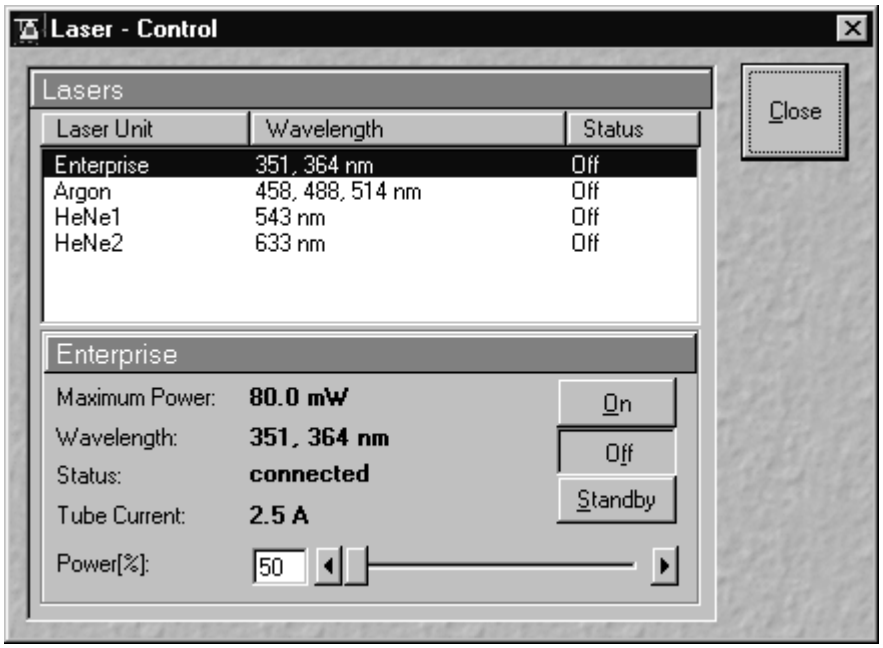


Fig. 4-9

(4) In the case of the argon and UV laser, click on the <Standby> button first.
Warming Up appears and, when the warming-up phase is finished, the **Ready** message. Then click on the <On> button: the laser is switched on.

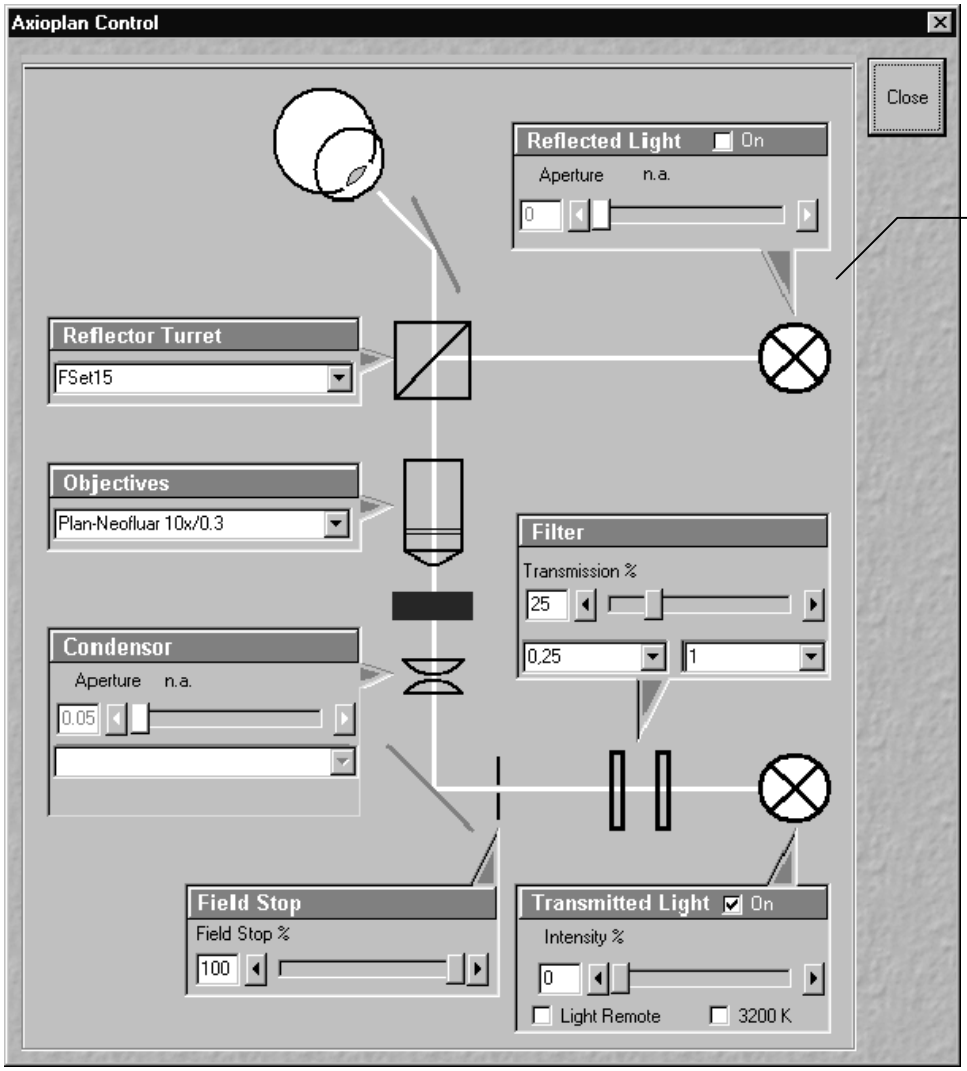
(5) Use **Power[%]** scrollbar to set the required laser power in %.

(6) In the case of the HeNe laser, click on the <On> button directly.

(7) Close menu.

4.3.4 Look in the Microscope and Visually Set Up Your Specimen

(1) Click on the <Micro> and <VIS> buttons from the LSM 510 tool bar.
The **Axioplan Control** menu or the **Axiovert Control** menu appears on the screen.



Dialog unit for
Microscope Axioplan

Fig. 4-10

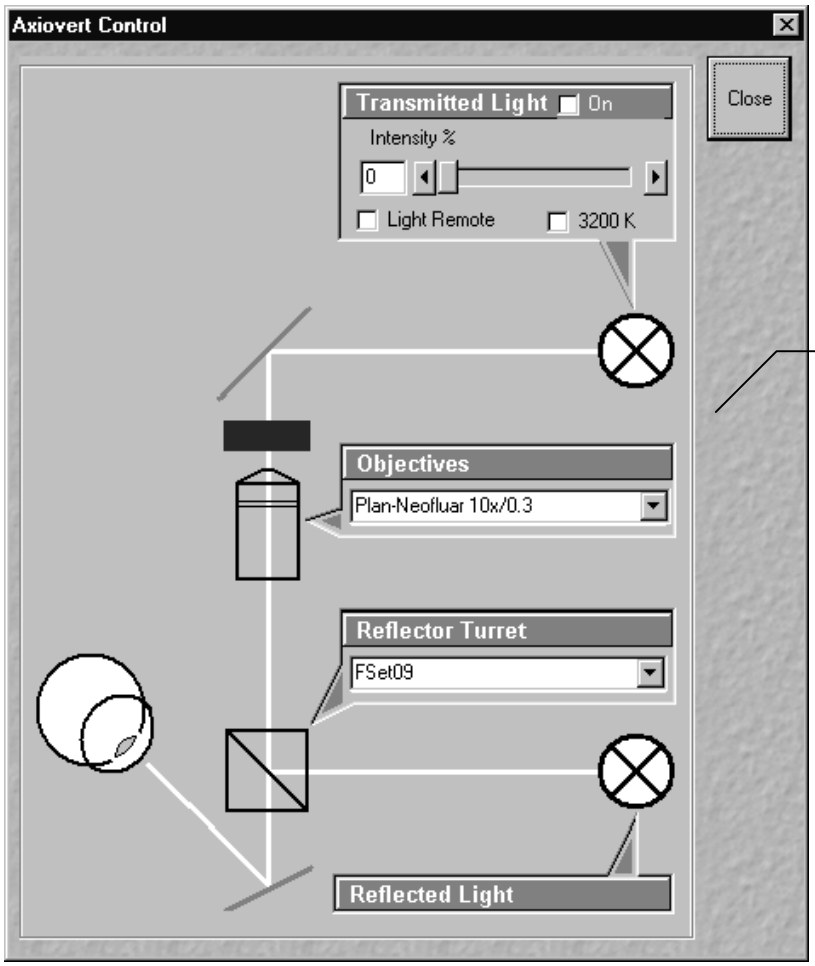


Fig. 4-11

(2) Move the silver slider on the side of the microscope to the appropriate position - the correct position will be shown in a message box on the monitor .

(3) Apply appropriate immersion fluid to objective if needed (see attached table for **Common Objective Characteristics**).
Select the Objective by clicking in the **Objectives** area of the **Axioplan Control** menu or **Axiovert Control** menu. Put specimen on the stage - make sure the specimen is mounted securely and flat.

(4) You can view the specimen in either fluorescence (reflected light) or transmitted light.

(5) To view in fluorescence click in the **Reflector Turret** area on the button and click on the Appropriate **FSET** (FSET 9 ≙ FITC, FSET 15 ≙ Rhodamine, FSET 01 ≙ DAPI) and set the check box for **Reflected Light On**.

(6) To view specimen in transmitted light set the **Relflector Turret** position to **None** and check box for **Transmitted Light On**.

(7) Note that there is a course/fine control button on the side of the microscope next to the focus knob.

(8) After the specimen is focused and the area of interest is selected, close the menu and click on the <LSM> button from the LSM 510 tool bar. The software will direct you regarding the appropriate setting of the silver slider. If required, push silver slider to the correct position.

4.3.5 Setting the Beam Path

(1) Click on the <Config> button from the LSM 510 tool bar.
The "Configuration Control" window appears on the screen.

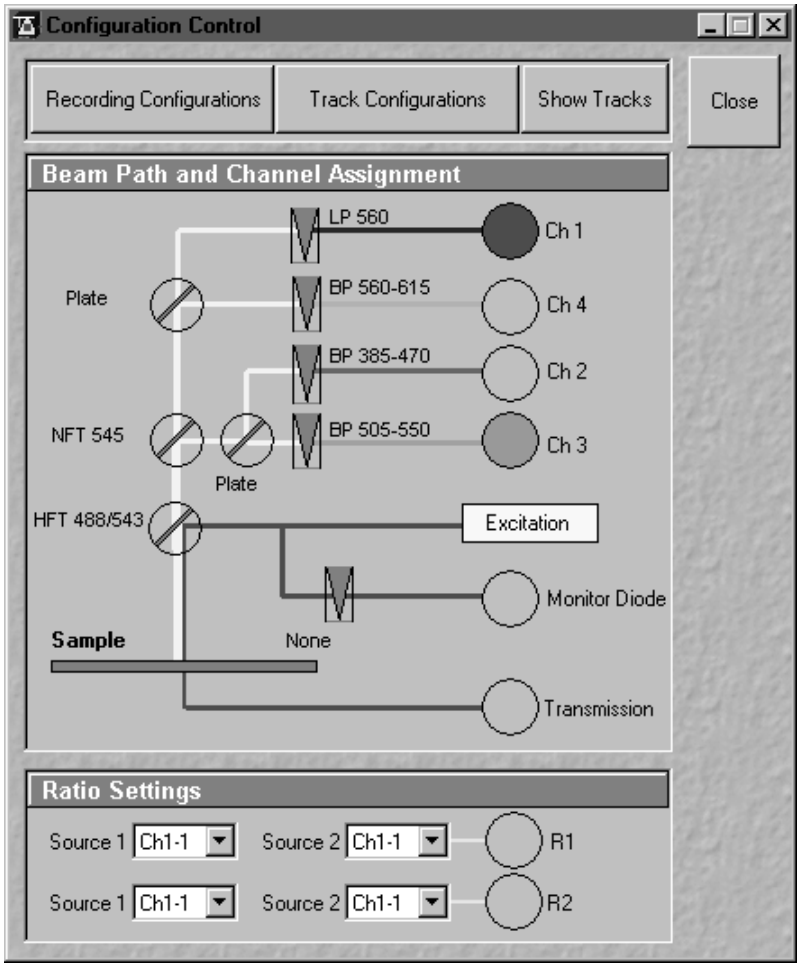


Fig. 4-12

(2) Click on the <Recording Configurations> button in the "Configuration Control" window. The "Recording Configurations" window appears on the screen.

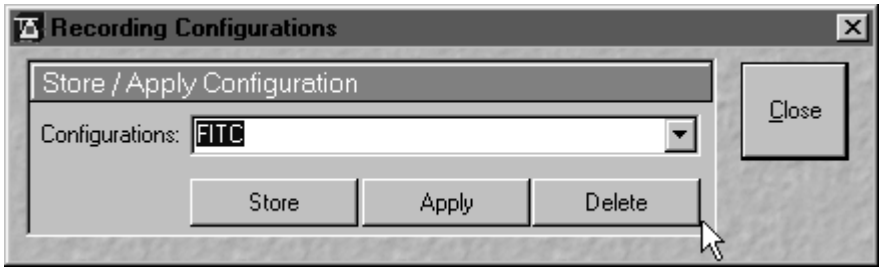


Fig. 4-13

(2) A list of configurations will appear by clicking on the button.
Choose a configuration from the list based on what you need to image from your specimen (e.g. FITC).

(3) Click on the <Apply> button.

(4) Settings will appear in the **Beam Path and Channel Assignment** portion of the menu.

(5) Close the menu.

4.3.6 Laser Scanning

(1) Click on the <Scan> button from the LSM 510 tool bar.
The "Scan Control" window appears on the screen.

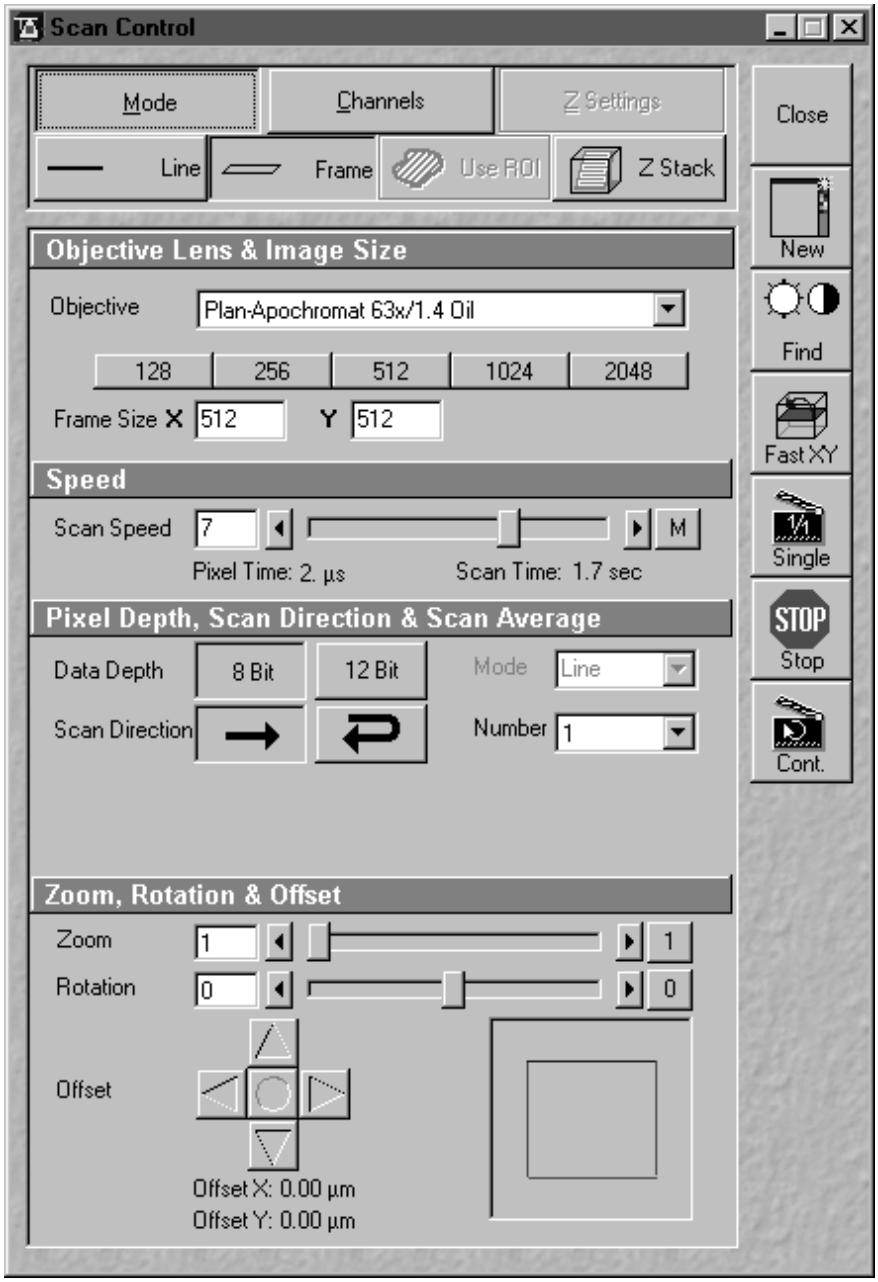


Fig. 4-14

(2) Click on the <Find> button on the right side of the **Scan Control** menu.

(3) Click on the <Single> button on the right side of the **Scan Control** menu.

(4) For multi-labeled specimens it is easier to view the image in Split screen where each label is arranged side by side.
The <Split> button is located on the right side of the image.

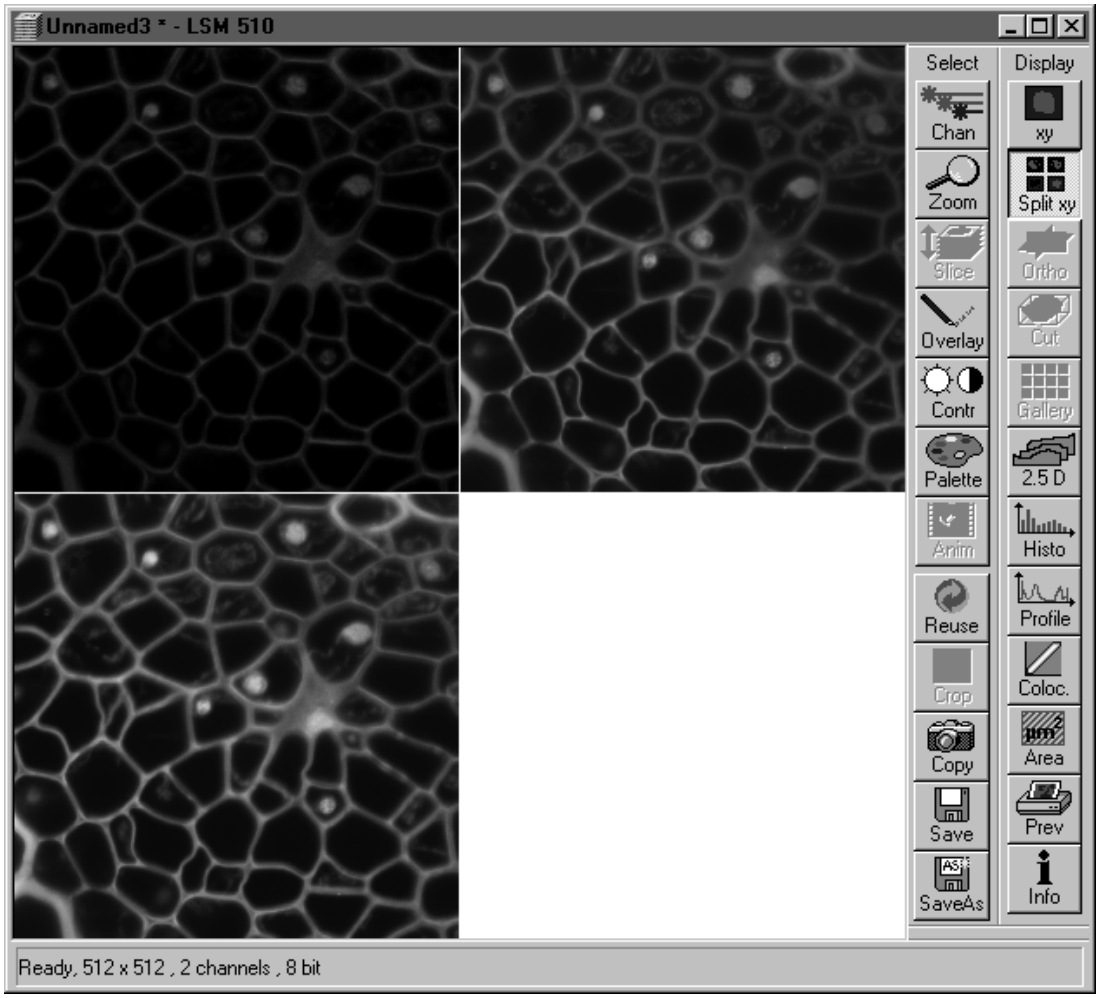


Fig. 4-15

(5) If you want to optimize the intensity of an image, you can adjust the detector gain (sensitivity) and Amplifier Offset (black level) as follows:

(6) Click on the <Channels> button from the **Scan Control** menu.

(7) Press on the <Cont.> (continuous scan) button on right side of the menu.

(8) Under **Channel Settings** you will see buttons for each channel you have set up.
Click **Ch 1-1**, for example, if you want to adjust the first image displayed in the split mode window.

(9) To make the image brighter or dimmer, adjust **Detector Gain**. This adjustment is very sensitive. Try using the left and right arrows to make the adjustment instead of dragging the slider bar.

(10) To adjust the black level (background) use **Ampl. Offset**.

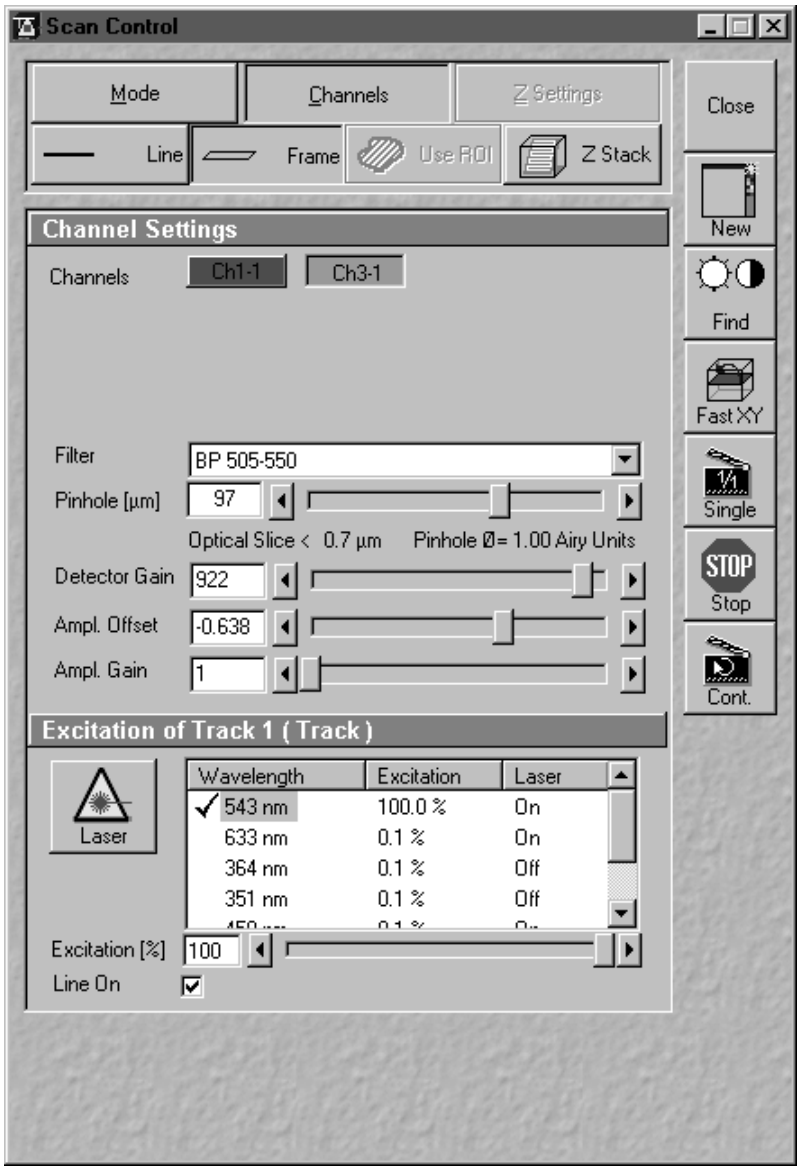


Fig. 4-16

(11) Also, try adjusting the microscope by manual focusing. Sometimes you will find that there are other focal planes within the specimen which are brighter, and therefore the detector gain will need to be turned down.

(12) Once you have optimized a particular channel, you can switch to the next channel desired and repeat steps 8, 9, and 10.

(13) As soon as all channels are optimized, click on the <Stop> button.

(14) To zoom into an area of interest click on the <Crop> button on the right side of the image. Zooming will enlarge an area of interest by scanning the laser into a smaller area of the field of view. This function can actually increase the X,Y resolution of an image.

(15) A red box will appear on the image.

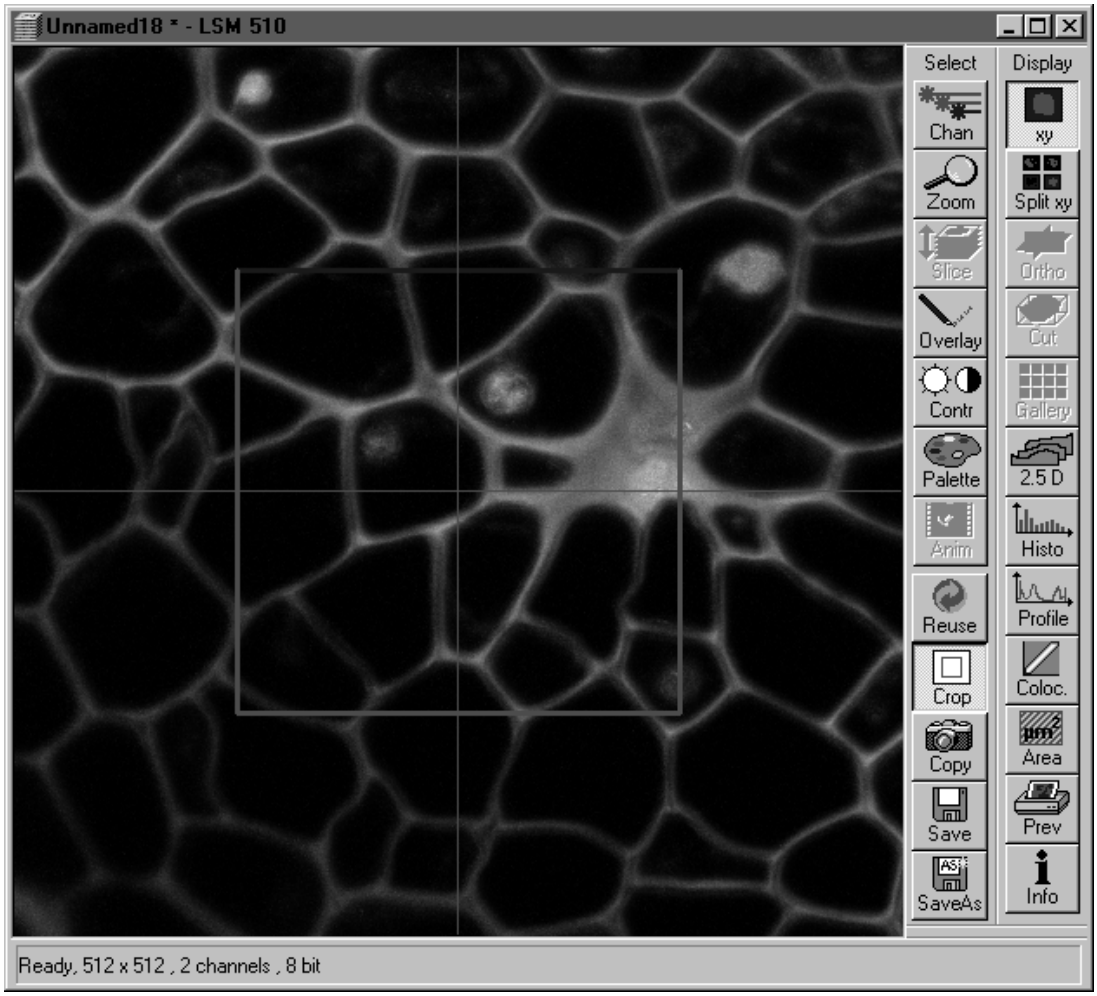


Fig. 4-17

Press and hold the mouse button on a corner of the red box and drag diagonally to resize the area. To move the entire box, just click into the box and move it to the required position by keeping the left mouse button pressed.

(16) Click on the <Cont.> button from the **Scan Control** menu and the image will now be zoomed up. You may have to readjust the **Detector gain** and **Ampl. Offset**.

(17) To further improve image quality you can slow the scan rate allowing more photons to integrate on the detector, or apply image averaging to remove random noise, or a combination of both. These adjustments are made by clicking on the <Mode> button on the **Scan Control** menu. Set the **Scan Speed** in the **Speed** area and **Number** in the **Depth, Scan Direction & Scan Average** area accordingly by observing your image. The setting average of 8 (Number 8) should improve signal/noise dramatically, however, the image acquisition rate will be slower.

(18) If your specimen is sensitive to photobleaching, you can attenuate the laser illumination by clicking on the <Channels> button from the **Scan Control** menu. At the bottom of the menu you can set the percentage of laser power (**Excitation %**) for each excitation wavelength. You will probably have to inrease the **Detector Gain** if you decrease the laser power. This setting controls the transmission degree of the AOTF.

(19) To save your image click on the <Save> or <Save As> button on the right side of the image.

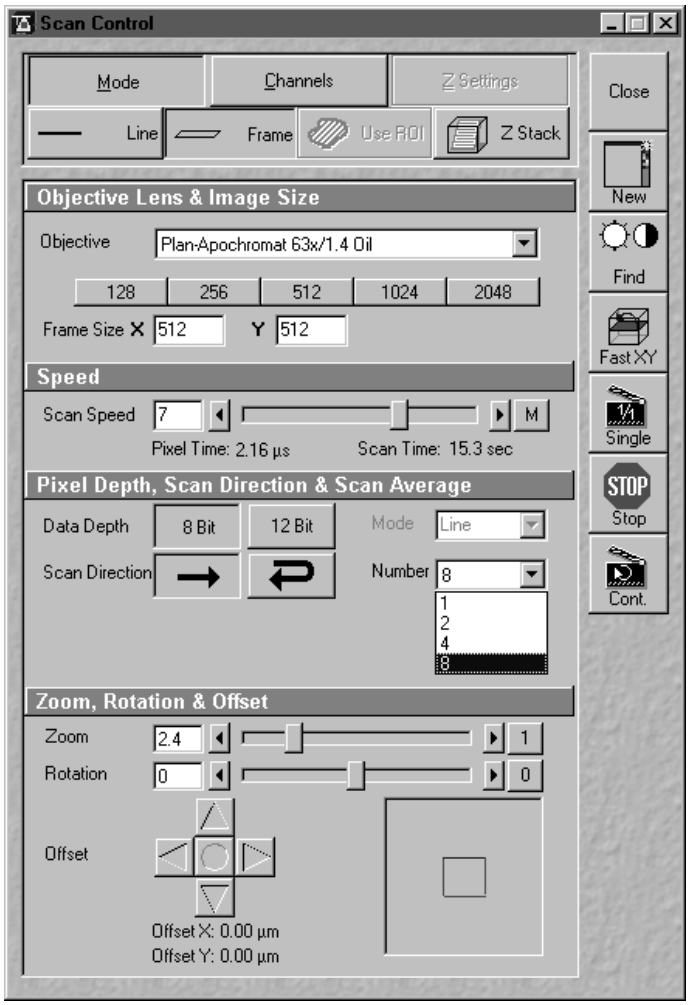


Fig. 4-18

4.3.7 Z Sectioning

Once you have set up your image as defined in the above section, you can collect a series of confocal images through the different focal planes of your specimen.

(1) Click on the <Z Stack> button on the **Scan Control** menu.

(2) If you have reduced the scan speed or have set image averaging, you should use the fast scanning mode to find the lowest and highest points of focus. These settings are made under **Mode** on the **Scan Control** menu, or directly via the <FAST XY> button.

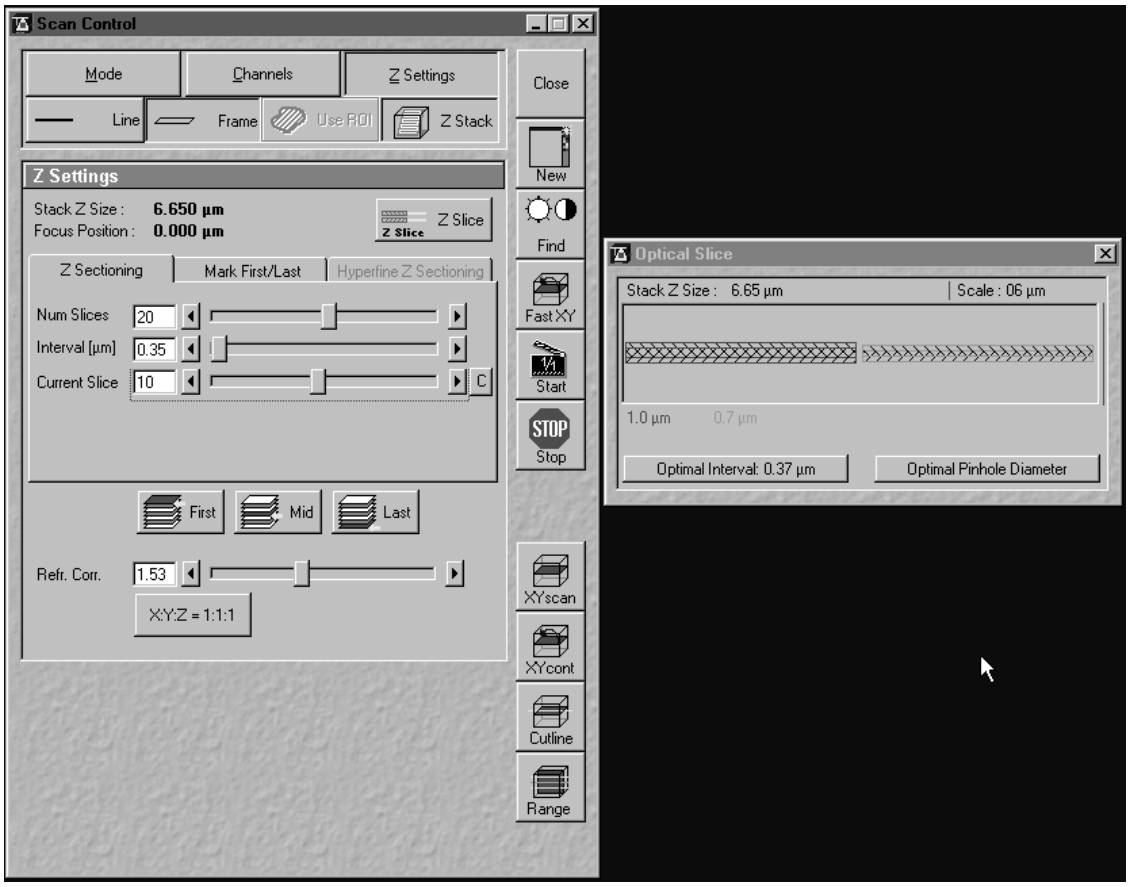


Fig. 4-19

(3) Click on the <Z Slice> button in the **Z Settings** panel.

(4) Click on the **Mark First/Last** register in the **Z Settings** panel.

(5) Click on the <XY cont.> button to begin scanning.

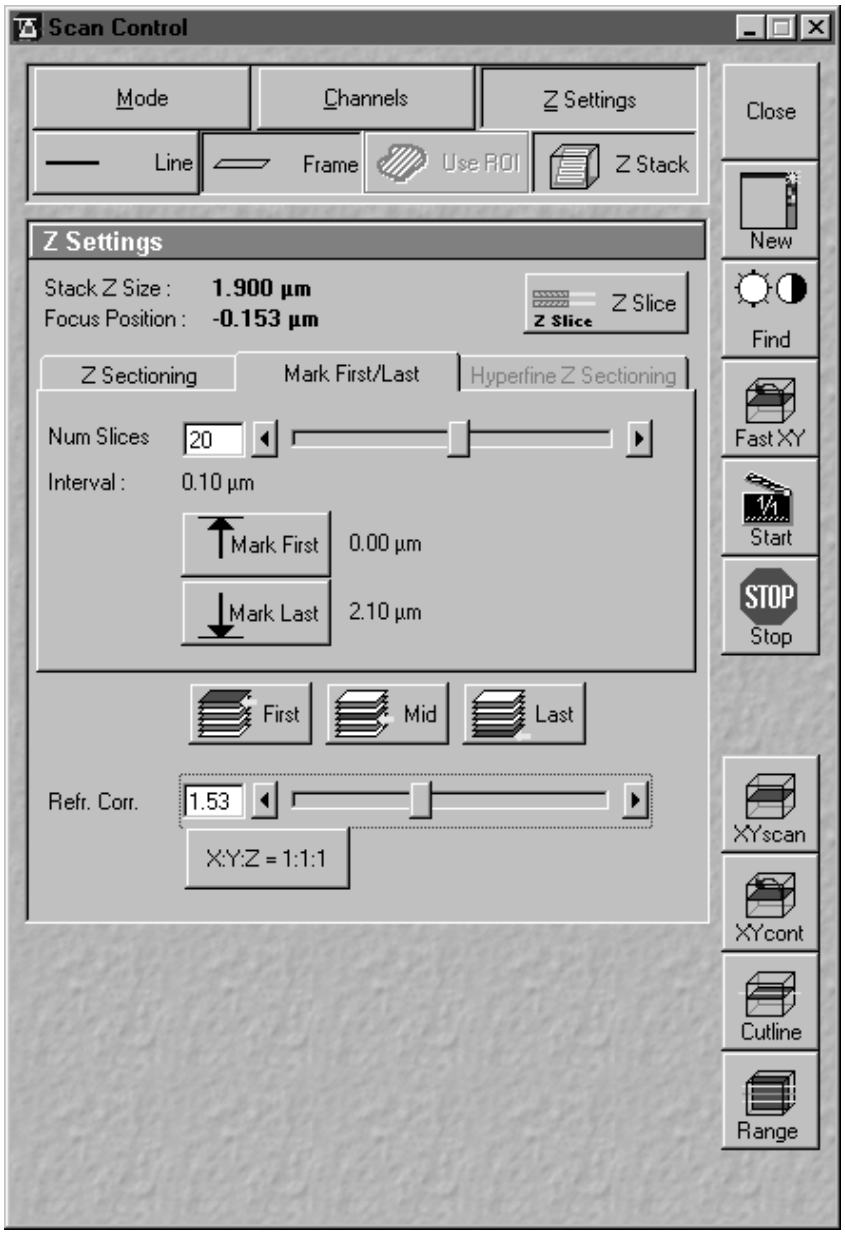


Fig. 4-20

(6) Move the focus down manually (clockwise) until the image of the specimen begins to disappear, then click on the <Mark First> button in the **Mark First/Last** register.

(7) Now move the focus in the opposite direction (up) until the image of the specimen begins to disappear.
Click on the <Mark Last> button in the **Mark First/Last** register.

(8) Click on the <Stop> button.

(9) Click on the <Optimal Interval> button in the "Optical Slice" window.
The optimum parameters are transferred to the **Z-Sectioning** section.

(10) Click on the <Start> button. The system will automatically start Z sectioning. Be careful not to bump the air table or the microscope until z sectioning is completed. Each successive z-slice can be viewed by changing to the **Gallery** Mode. This button is located on the right side of the image.

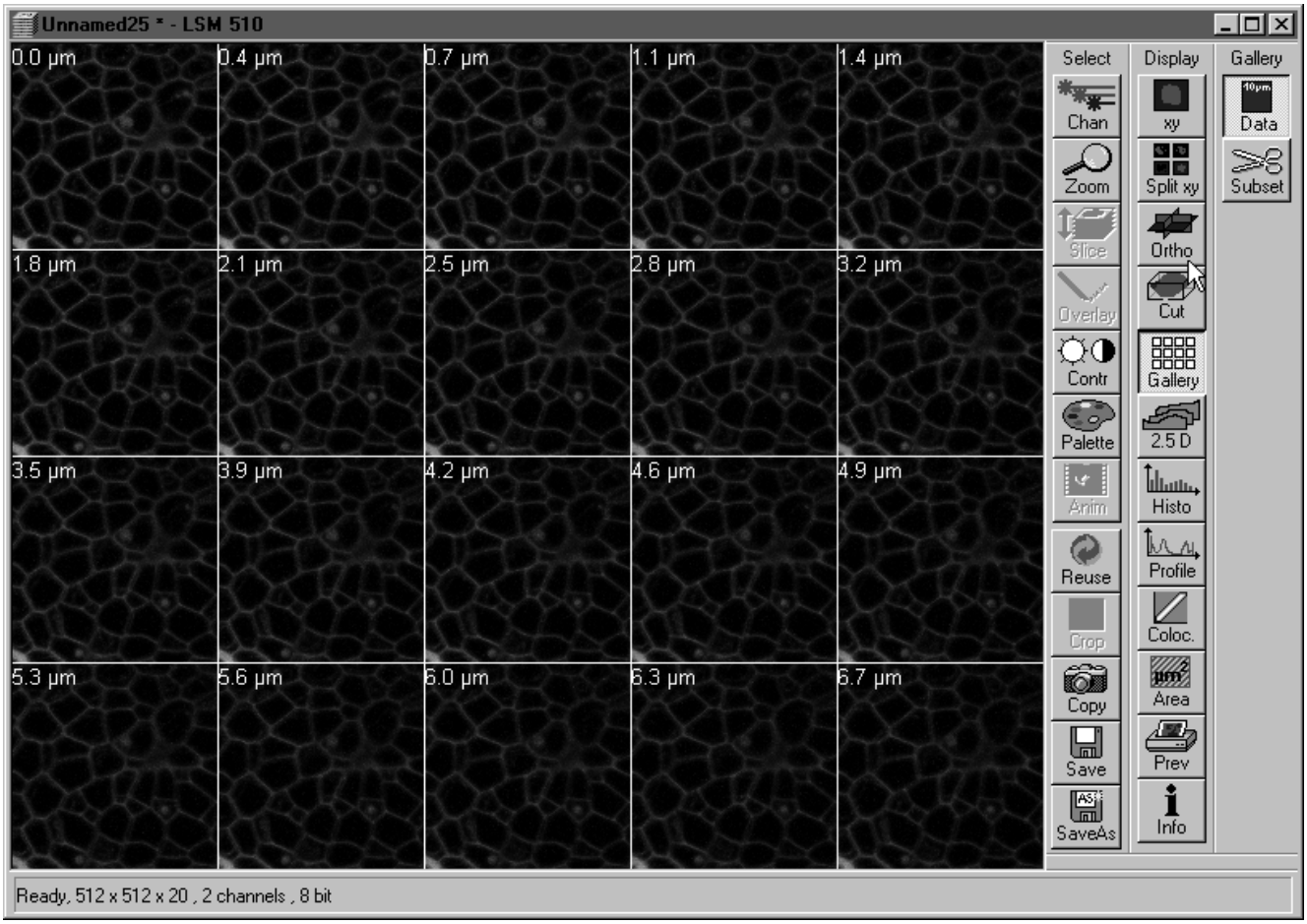


Fig. 4-21

(11) A black bar will be shown under the image and will move from left to right, showing that the LSM 510 is in the process of Z sectioning. The laser will automatically stop scanning when z-sectioning is completed.

(12) The entire stack of images can be saved using the <Save> or <Save As> buttons on the right side of the image.

4.4 Overview of the Menu Items

- Start the LSM program as follows:
  - Double-click on the **Start LSM 510** icon on the desktop.

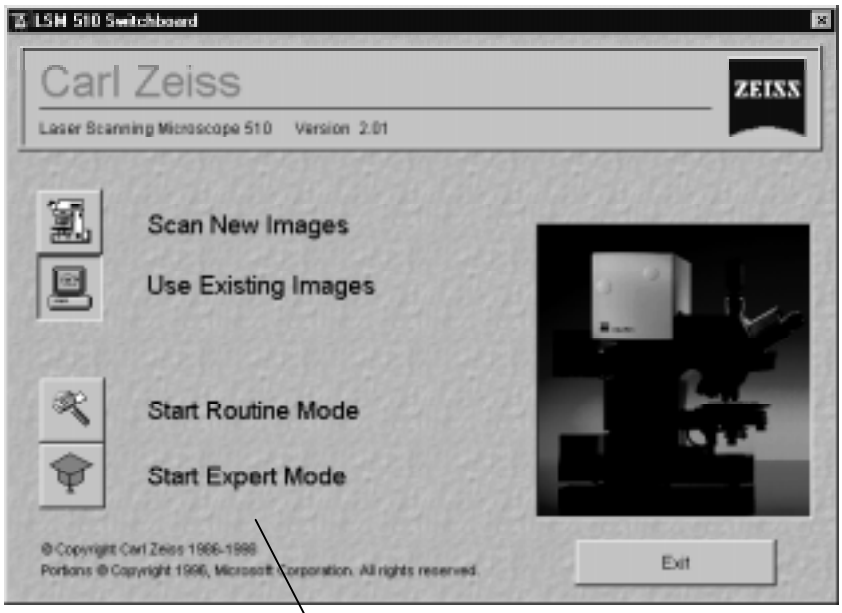


Fig. 4-22 LSM 5 Switchboard menu

The switchboard menu presents the following items for selection:

- Scan New Images
  Clicking on this button activates the complete LSM hardware.
- Use Existing Images
  This item allows you to process and analyze previously acquired images with the LSM software. In this mode, control of the hardware (laser module...) is not possible.

☞ Please note that the <Scan New Images> button must be activated before setting up the Routine Mode or the Expert Mode. Otherwise, the hardware can not be controlled by the LSM software.

Operating modes

- Start Routine Mode
  Click on this button if you want to work with pre-configured system settings (typical applications).

- Start Expert Mode
  Use of this mode requires that you are thoroughly familiar with the exact microscope procedures and interrelations.
  You need to set all parameters and functions upon your own decision; this mode therefore provides you with the greatest flexibility of operation.
  It is also possible, however, to call up stored configurations and to modify the parameters/settings if necessary.

☞ **Start LSM Program:**
Some printers (for example KODAK Thermo Printer) will produce an error message "hard key not found" in case the printer is not powered on.
Fix: turn on the printer before starting the LSM software.

Don't switch off the KODAK printer during the scanning process.

- Click on the <Start Expert Mode> button.
  - The "LSM 510 - Expert Mode" main menu appears on the screen.

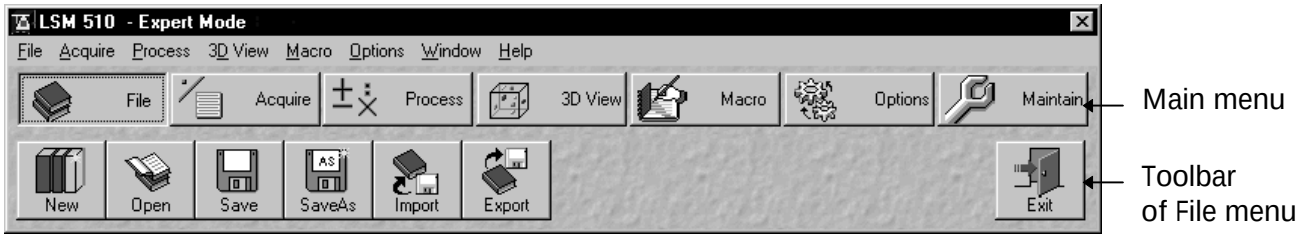


Fig. 4-23

  - The <File> button is active automatically, and the submenus selectable in it are shown in the second (bottom) toolbar.

The buttons of the main menu (upper toolbar) have the following meanings:

File	Open, save, import and export of image data.
Acquire	Calling up and setting the necessary operating parameters. During the preparation for, and execution of, laser scan image acquisition, this menu item is used as the working dialog between the computer and the microscope.

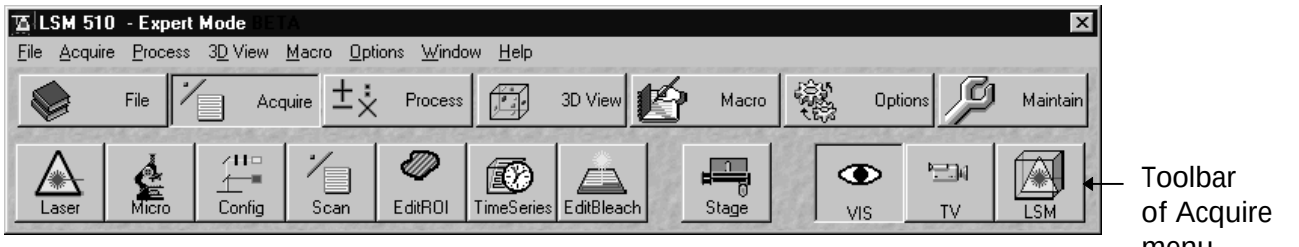
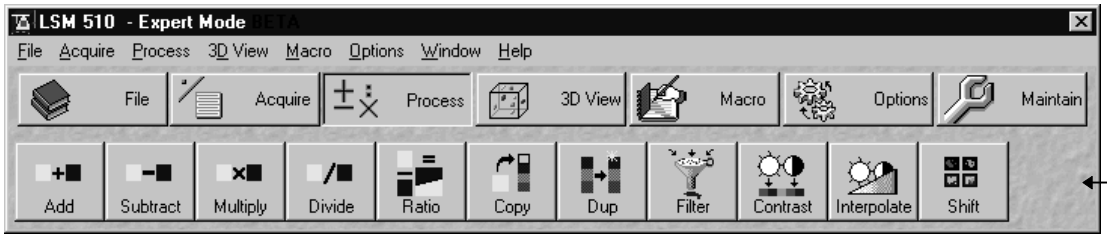


Fig. 4-24

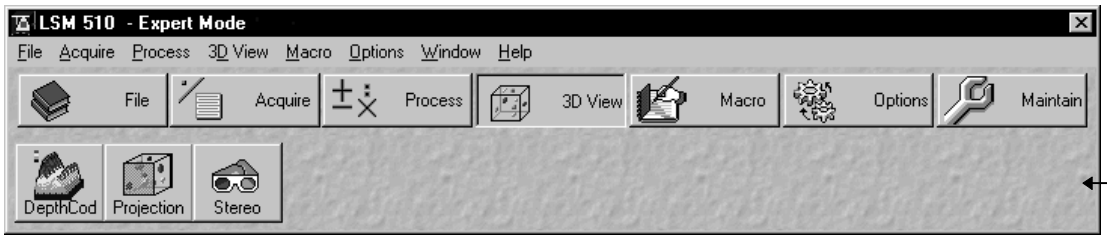
Process — Used for the mathematical integration of acquired images.
<Shift> allows you to perform a pixel shift correction.



Toolbar of Process menu

Fig. 4-25

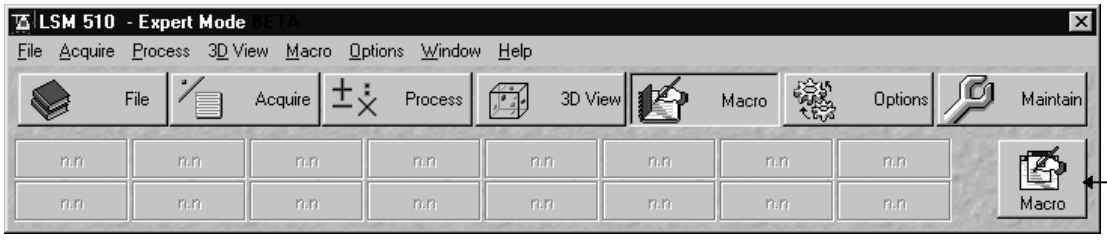
3D — For three-dimensional image processing.



Toolbar of 3D View menu

Fig. 4-26

Macros — Makes it possible for the user to store frequently used processes and to run them automatically.



Toolbar of Options menu

Fig. 4-27

Options

For custom-configuration of software and hardware options, and for exporting system operating sequences to the Routine Mode.

Under this menu item access to the colouring table will be enabled.

In the "Settings for User" window you can specify essential operating modes and informative help, organized by registers, which have an effect on the user interface.

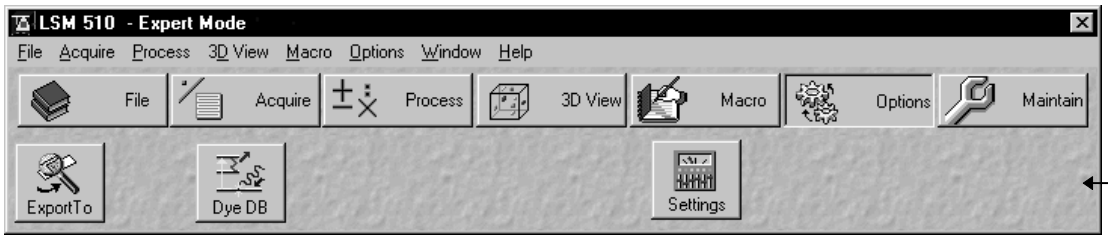


Fig. 4-28

Maintain

This is a service mode for the adjustment and setting of other parameters (e.g. objectives).

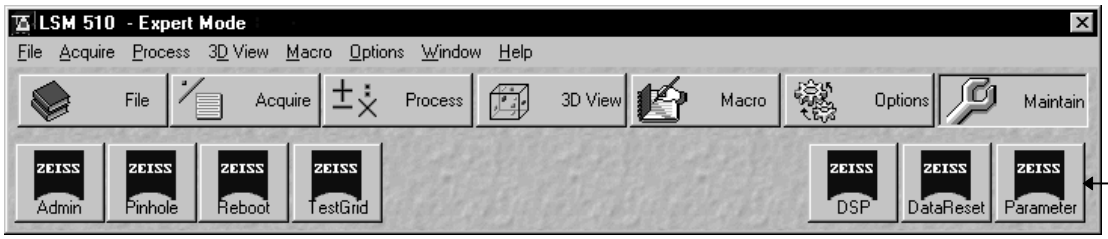


Fig. 4-29

4.5 Image Acquisition (Acquire)

- In the main menu toolbar, click on <Acquire>.
  - This opens another, subordinate toolbar in the main menu.

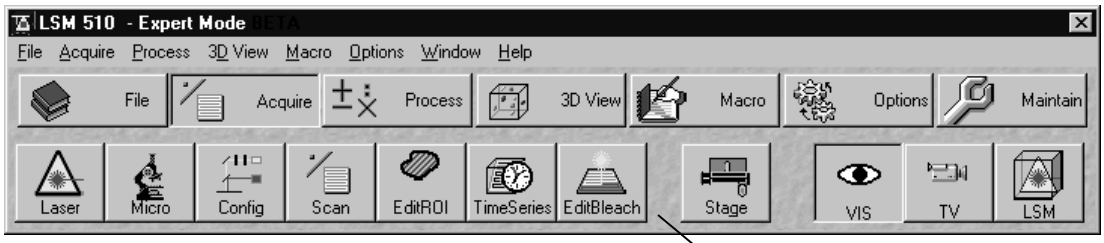


Fig. 4-30

☞ For preparing and acquiring a scanning image, it is recommended to call up and use the tools of the subordinate toolbar in succession from left to right.

- Laser setting.
- Conventional microscope setting.
- Configuring the optical system for the Scanning Mode.
- Setting of scan parameters.
- <EditROI> permits up to 10 areas within a frame to be defined and scanned.
- <TimeSeries> permits user-specific time series to be selected for the scan procedure.
- The <EditBleach> function is used to bleach a defined, freely selectable area within the scanning field.
- Upon selecting <Stage> you can set the focus (Z coordinate) and the Z step size between successive slices. If the optional, motorized X/Y-stage is connected, the X and Y-positions of the sample can also be selected.
- With the <VIS>, <TV> and <LSM> buttons you can effect a PC-supported switching of the beam path.
- VIS for conventional microscopy through the eyepieces.
- TV conventional microscopy for observation by means of a TV camera.
- LSM Laser scanning mode.

☞ For the scanning process, the <LSM> button in the toolbar subordinate to the "Acquire" item must be activated, and the silver slider on the microscope must be in the LSM position.

4.5.1 Laser settings

- Click on the <Laser> button.
  - This opens the "Laser Control" window, which shows all lasers connected to the system.

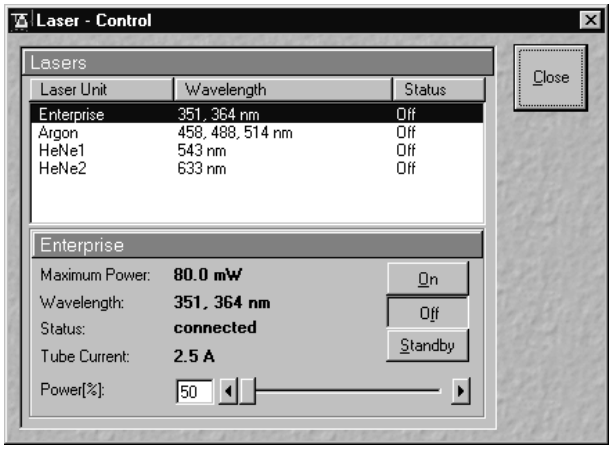


Fig. 4-31

The **Laser Selection and Power Status** panel, shows the types, operating statuses and excitation wavelengths of the lasers available.

- Click on the desired laser on the **Laser Selection and Power Status** panel.
  - This highlights the selected laser.

On the "Laser Control" window, activate the laser as follows:

This applies to Coherent UV-Laser 653 II (Enterprise) and Ar-multiline Laser:

- Click on the <Standby> button.
  - Wait for the laser to heat up, until the Status ready - Standby message appears.
- Click on the <On> button.
  - Status ready - On appears.
- Use the Power [%] slider to set the laser power which is ideal for the measurement job. To do this, click on the slider and drag it while keeping the left mouse button depressed.

  To change the laser power in steps of 1%, keep the <Ctrl> key depressed while clicking the mouse on the ◀ or ▶ arrow buttons.

Thus, the laser needed for image acquisition is available.

Enterprise: Set power between 50 and 100 % of the maximum tube current.
Optimum operation is at 20 A (Tube Current [A]).

Argon: Set power between 25 and 100 % of the maximum tube current.
Optimum operation is at 8 A.

To switch on the Enterprise laser, proceed as follows:

(1) The internal water cooling LP 5 is running.

(2) Start the PC, wait until NT system is booted.

(3) Switch on the Power Supply of the Enterprise laser, power potentiometer turned to maximum.

(4) Start the LSM 510 software.

If the LSM 510 software is already running and you want to use the UV laser, do the following:

(1) Close the LSM 510 software.

(2) Switch on the power supply of the Enterprise / power potentiometer turned to maximum.

(3) Start the LSM 510 software again.

This applies to HeNe lasers:

- After selecting the laser, click on the <On> button.
  - After a short heat-up phase of about 15 seconds, "Status ready - On" appears.
  - The required laser for image acquisition is now available.
- Click on the <Close> button to close the "Laser Control" window.

4.5.2 Microscope settings (conventional microscopy)

- Place specimen on microscope stage.
  - The cover slip must face up on an upright microscope, down on an inverted microscope.
- Select the objective to suit the job - dry, water or oil immersion (see inscriptions on objectives: w for water and oil for immersion oil).
- Click on the <Micro> button.
  - This opens the "Microscope Control" window on the screen.

If you are using the Axioplan 2 MOT, the "Microscope Control" menu shown in Fig. 4-32 appears with the title "Axioplan Control".

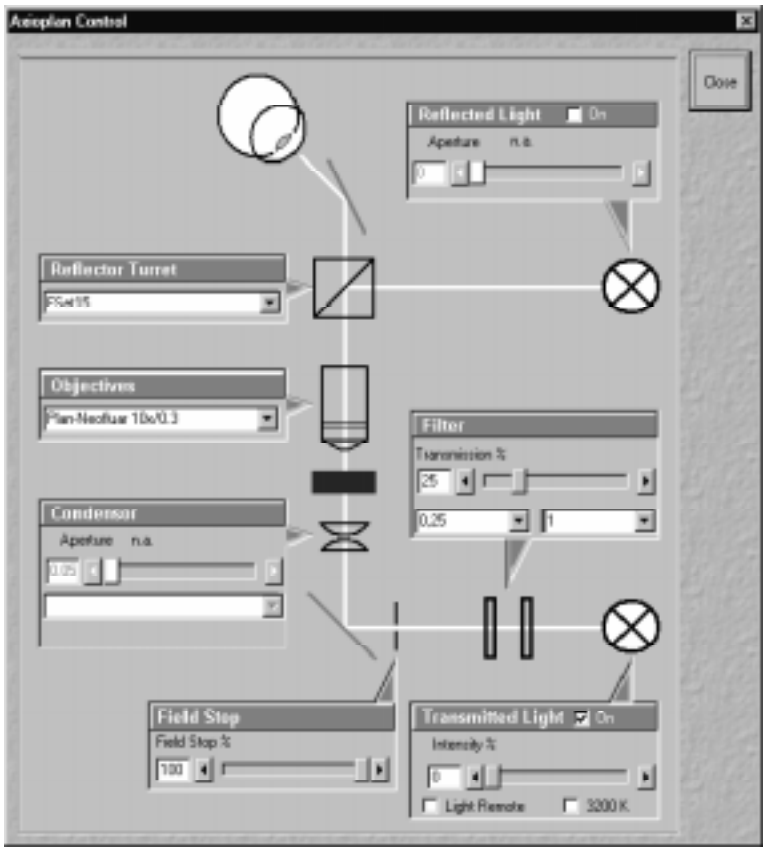


Fig. 4-32

If you are using the Axiovert 100 M, the "Microscope Control" menu shown in Fig. 4-33 appears with the title "Axiovert Control".

With **Transmitted Light** activated  , the halogen lamp is automatically occluded in the laser scanning mode.

Please bear in mind that the light intensity does not automatically correspond to 0 % when **Light Remote** is deactivated. The microscope setting (light intensity) of the last session, which was not remote-controlled, is restored on exit of the program.

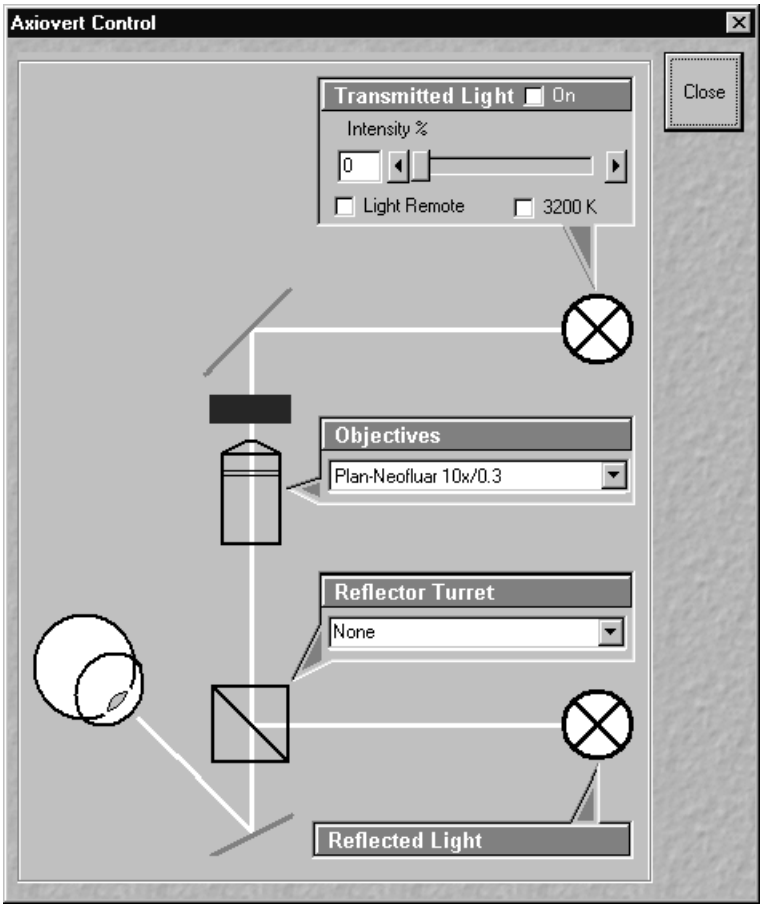


Fig. 4-33

- In the **Objectives** list box, select the required objective as follows:
  - Open the list box.
  - Click on the objective you want to select.
  - The selected objective will automatically move into the beam path.

☞ Note that the nosepiece of the Axiovert 100 M microscope must not be moved manually. To bring another objective into the beam path, move the objective nosepiece only software-controlled.

If you want to use an objective which is not contained in the nosepiece, proceed as follows:

(1) Change objectives

(2) Click on the "Change Objectives" icon. The submenu "Modify Objectives Settings" appears.

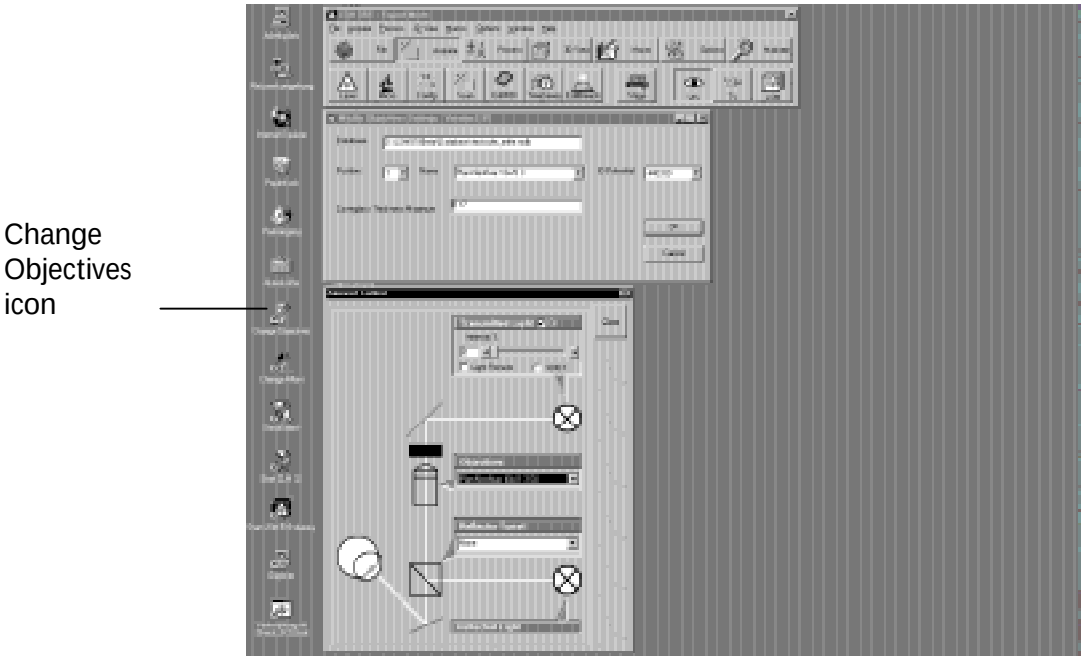


Fig. 4-34

(3) Choose the objective corresponding to the desired position.

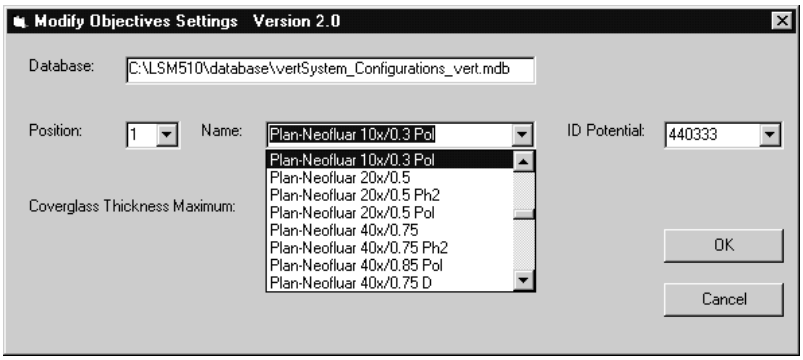


Fig. 4-35

(4) Click on the <Maintain> button in the main menu and then on the <Reboot> button in the submenu.

(5) Confirm the selection in the "Reboot Components" dialog box by clicking on the <Reload Objectives from DB> button.

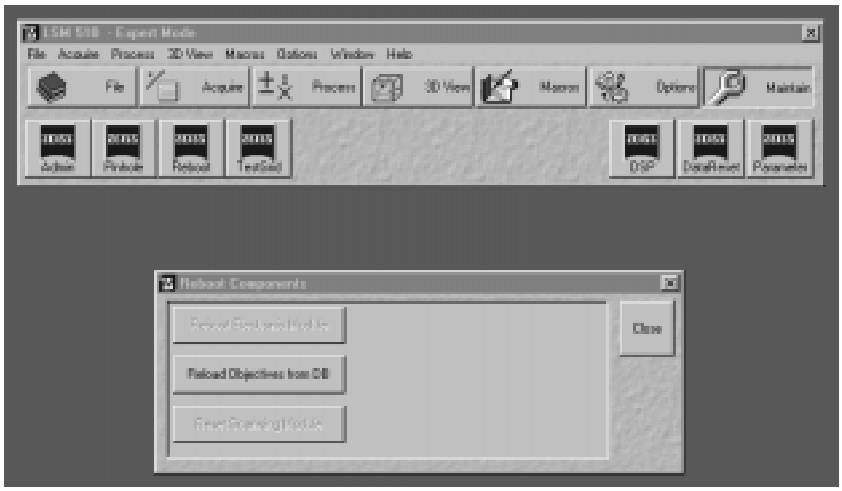


Fig. 4-36

4.5.2.1 Transmitted-light observation (Axioplan 2 MOT)

- Push in the silver slider (4-37/8) on the microscope tube as far as it will go.
  - This opens the light path for specimen observation through the eyepieces.
- Actuate the shutter switch (4-37/4) to open the light path of the halogen lamp, and control its brightness with the potentiometer (4-37/3).
- Use the focusing drive (4-37/5) to focus the required object plane.
- Select specimen detail by moving the stage in X and Y using the XY stage fine motion control (4-37/6 and 7).

1 Occluding slider
2 Switch, HBO 100 power supply
3 Brightness control potentiometer
4 Shutter switch: transmitted/reflected light
5 Focusing drive
6 Stage fine motion control, X
7 Stage fine motion control, Y
8 Silver slider

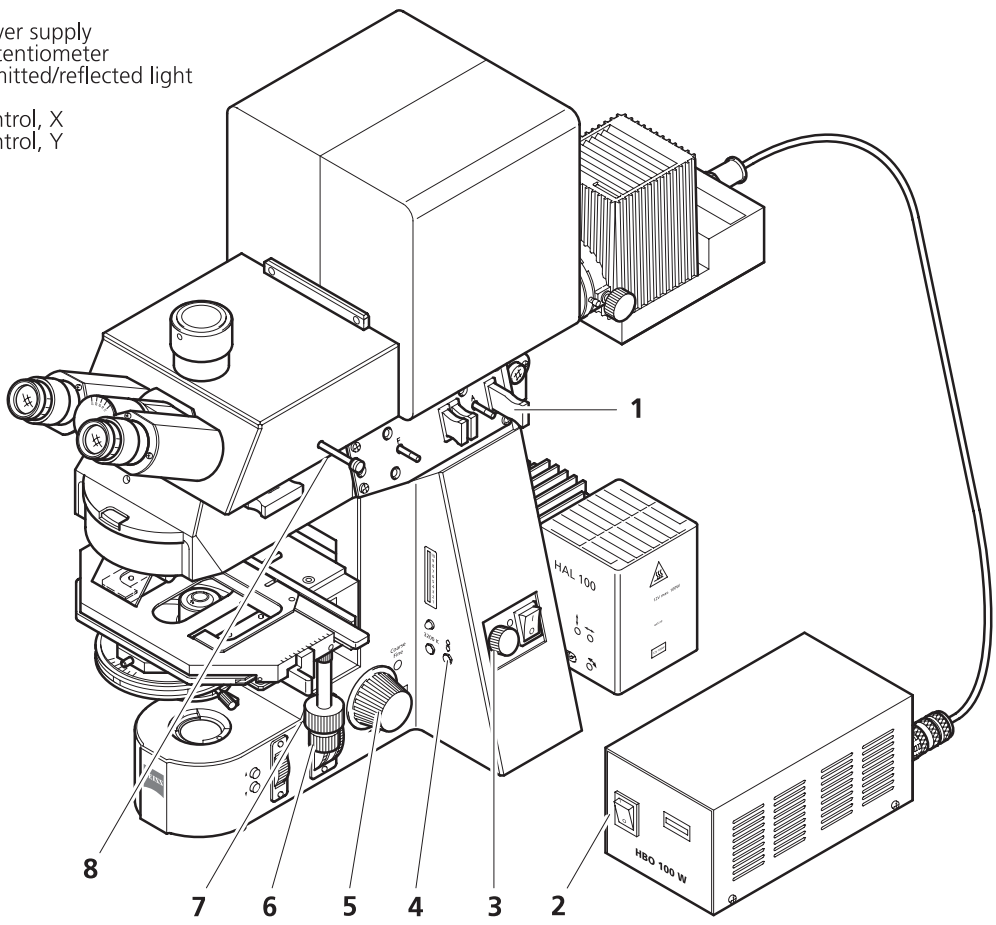


Fig. 4-37

4.5.2.2 Epi-fluorescence observation (Axioplan 2 MOT)

- Turn on the HBO 100 W power supply with switch (4-37/2).
- Push in the silver slider (4-37/8) on the microscope tube as far as it will go.
  - This opens the light path for specimen observation through the eyepieces.
- Switch on the reflected light.
- Pull out the occluding slider (4-37/1) to a light-passing position; actuate shutter switch (4-37/4) for reflected light if it is in transmitted-light position.

☞ To avoid excessive bleaching, expose the specimen to the minimum possible irradiation, i.e. keep the irradiation time as short as possible.

- In the **Reflector Turret** list box, select the reflector module (filter sets) to suit the type of fluorescence excitation. Proceed as follows:
  - Open the **Reflector Turret** list box.
  - Click on the desired reflector module.
  - The reflector turret moves the selected reflector module into the beam path.

☞ The FITC filter set (FSET09) consists of an excitation filter for the 450 - 490 nm spectral range, an FT color splitter for 510 nm and an LP long pass filter, which passes emission light wavelengths greater than 510 nm.

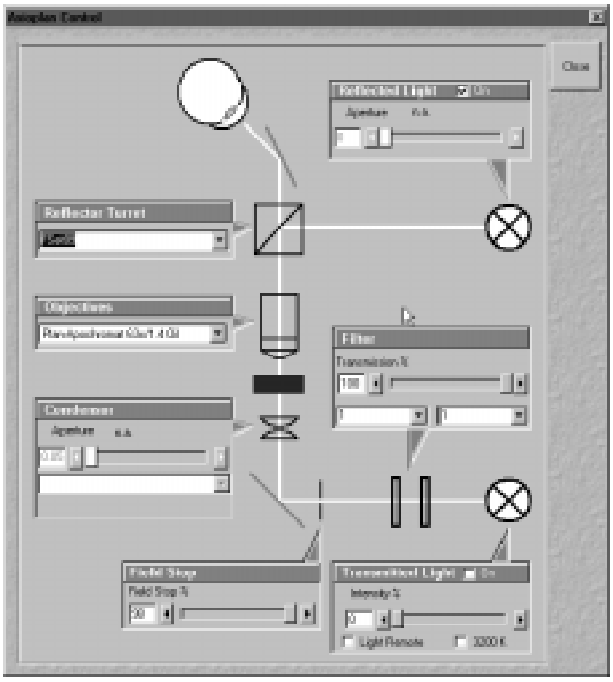


Fig. 4-38

Other filter sets:

The filter sets described in this section are included in the standard configuration, but other sets are available on request.

DAPI:	BP 365	FSET01
	FT 395	
	LP 397	
TRITC:	BP 546	FSET15
	FT 580	
	LP 590	

- Use the focusing drive (4-37/5) to focus the required object plane.
- Select specimen detail by moving the stage in X and Y via the XY coaxial drive (4-37/6 and 7).

This completes the conventional setting of the microscope before starting the laser scanning of the specimen.

- Click on the <Close> button to close the "Microscope Control" window.

4.5.2.3 Transmitted-light observation (Axiovert 100 M BP)

- Pull out the silver slider (4-39/1) on the microscope tube as far as it will go.
  - This opens the light path for specimen observation through the eyepieces.
- Push in the sideport slider (4-39/2) as far as it will go.
- Pull out the VIS slider (4-39/3) as far as it will go.
- Use the focusing drive (4-39/7) to focus the required specimen plane.
- Select specimen detail by moving the stage in X and Y via the XY stage fine motion control (4-39/6 and 5).

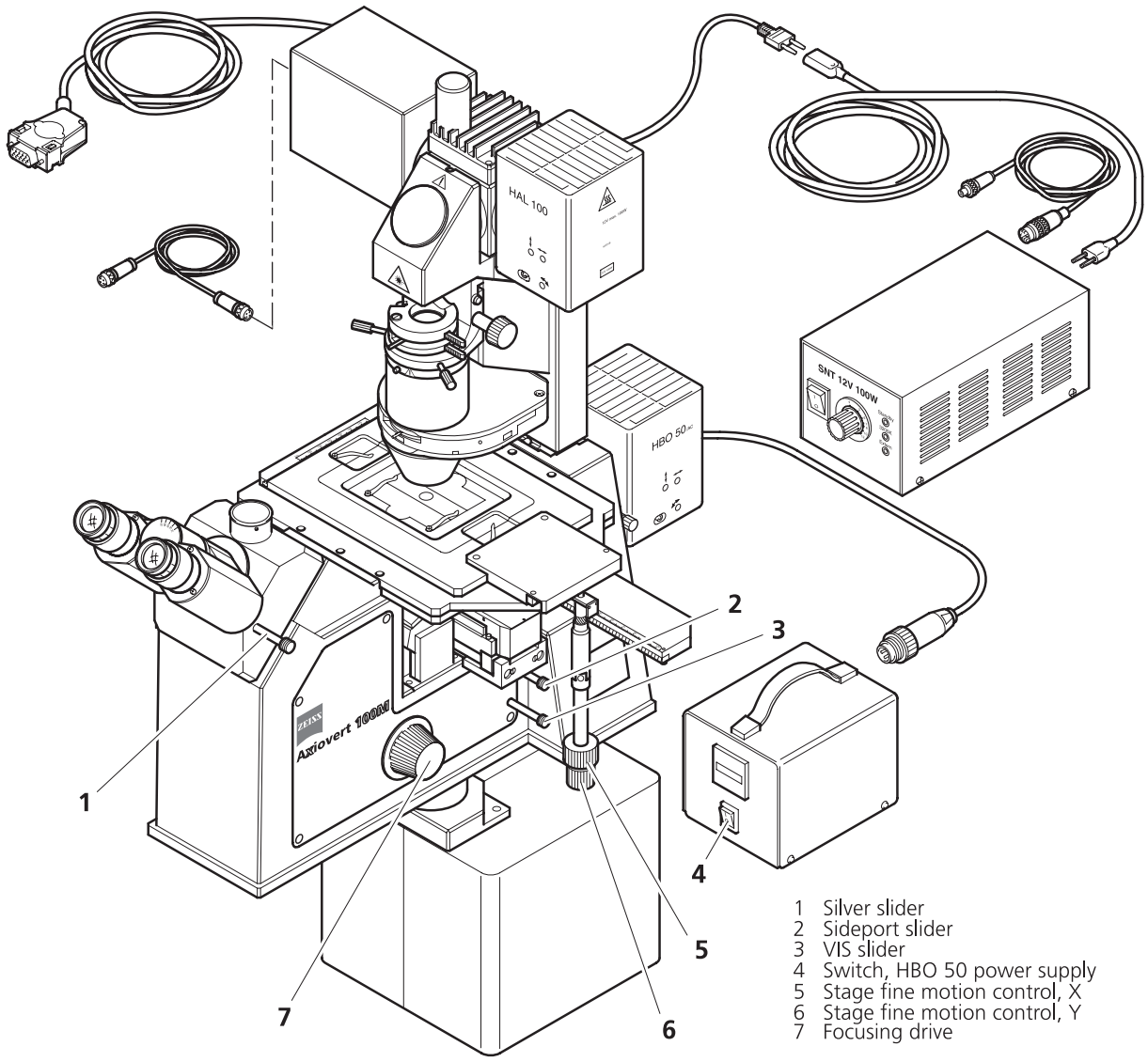


Fig. 4-39

4.5.2.4 Epi-fluorescence observation (Axiovert 100 M BP)

- Turn on the HBO 50 power supply switch (4-39/4).
- Pull out the silver slider (4-39/1) on the microscope tube as far as possible.
  - This opens the light path for specimen observation through the eyepieces.
- Push in the sideport slider (4-39/2) as far as possible.
- Pull out the VIS slider (4-39/3) as far as possible.
- In the **Reflector Turret** list box, select the desired filter set by clicking on it.

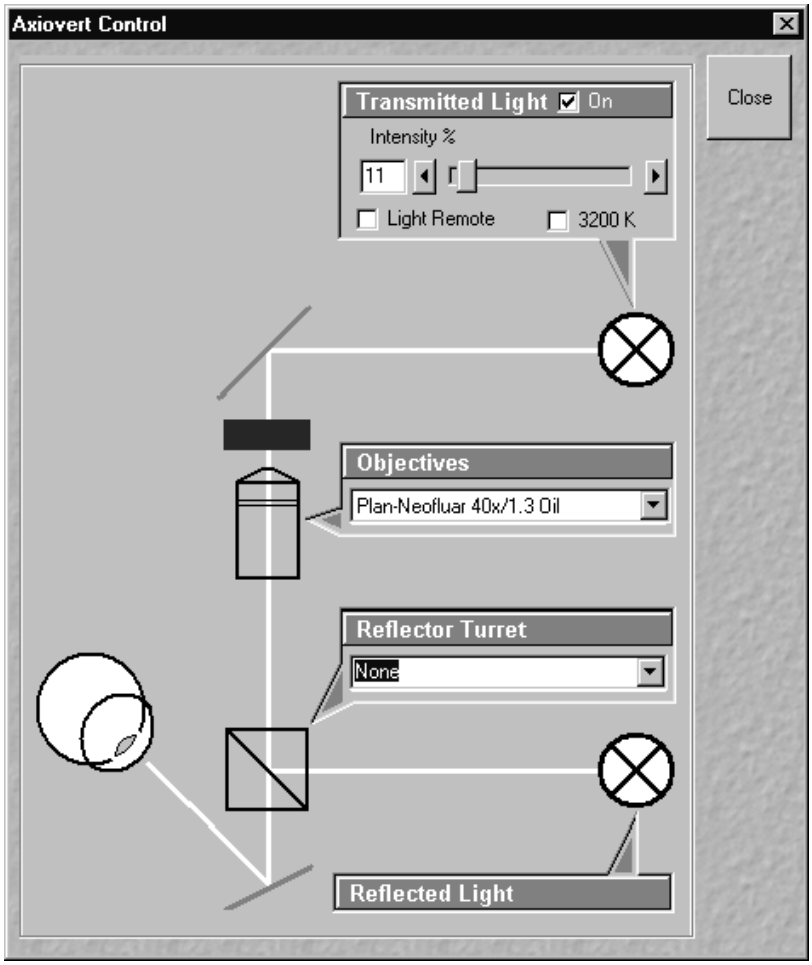


Fig. 4-40

The filter is automatically moved into the beam path to enable observation in epi-fluorescence.

- In the **Objectives** list box, select the objective.

4.5.3 Beam path / Configuration

- Click on the <Config> button.
  - This opens the "Configuration Control" window, in which you can configure the system for scanning. The window contains two panels: **Beam Path and Channel Assignment** and **Ratio Settings**.

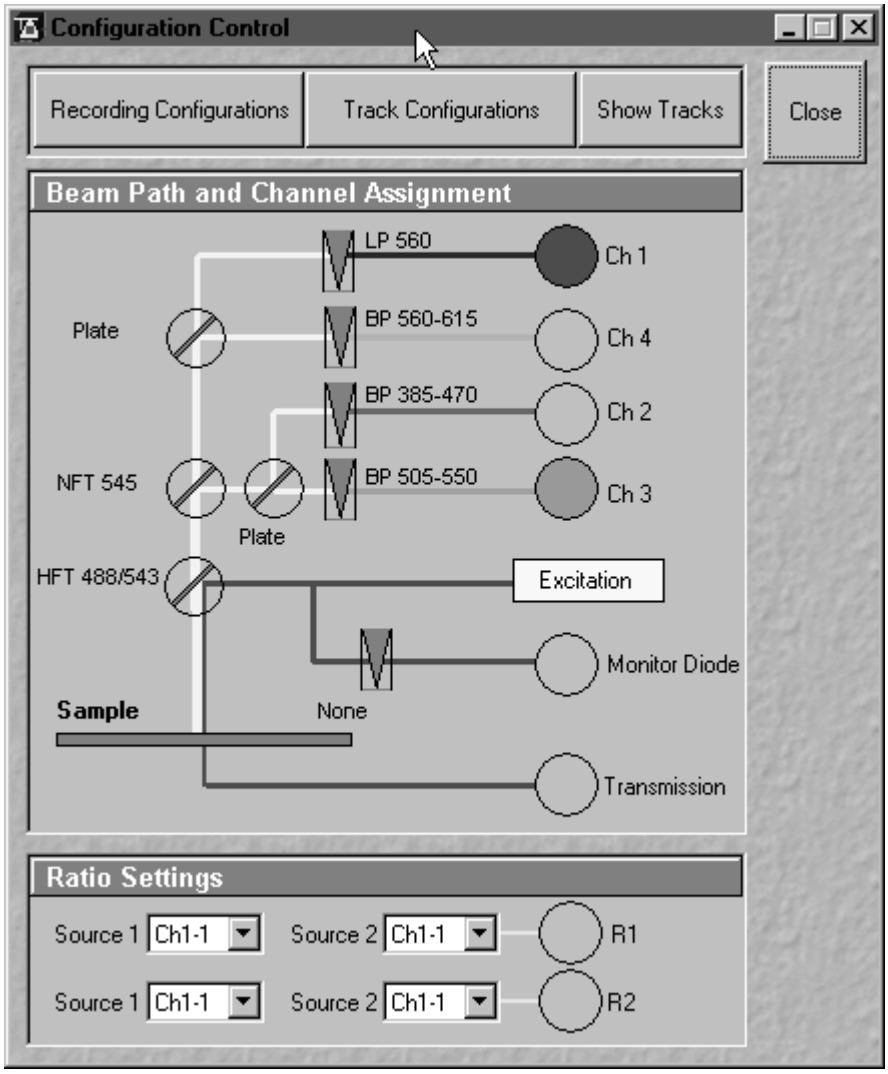


Fig. 4-41

☞ The **Beam Path and Channel Assignment** panel differs according to the hardware configuration supplied.

4.5.3.1 Taking over the configuration with Configuration Control

After clicking on the <Recording Configurations> button, the **Recording Configurations** dialog box appears on the screen and allows the following settings to be made:

- Selection and calling up a configuration, whether factory-preset or created by the user on the **Store / Apply Configuration** panel
- Saving a user-created configuration under a freely selectable name
- Deletion of a configuration

A configuration stored in the database, whether factory-supplied or user-created, can be accepted or selected for active operation as follows:

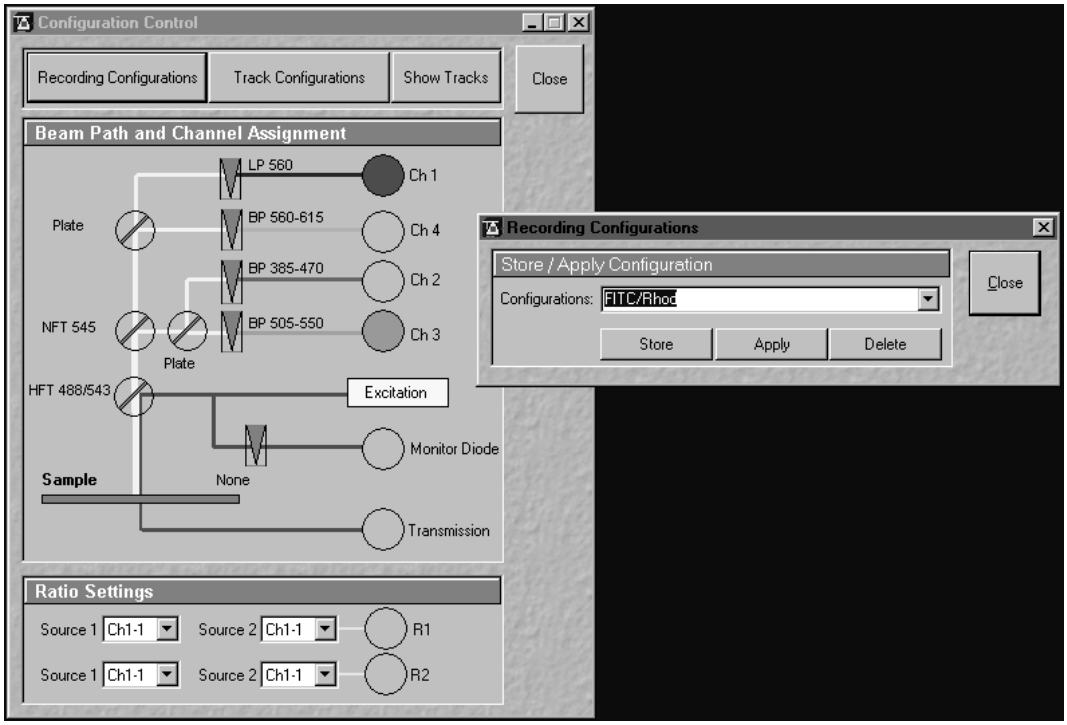


Fig. 4-42

- On the **Store / Apply Configuration** panel, click on the arrow button .
  - This opens a drop-down list of all stored configurations.
- Browse through the configurations by clicking, or use the scroll bar at the side of the drop-down list.

- Click on the desired configuration.
  - The selected configuration is shown in the **Configurations** status box.

Click on the <Apply> button.

  - This results in the stored instrument parameters being taken over for active use.

☞ The optical diagram of the configuration selected appears on the **Beam Path and Channel Assignment** panel.

4.5.3.2 Tracks function

If the existing four channels are not sufficient for image acquisition (use of more than four excitation laser wavelengths or recording of more than four emission ranges), it is possible to use eight channels or the maximum of four tracks in one scanning procedure. If a second track or further tracks are used, the scan parameters can be changed as required. This allows "cross-talking" from one channel to another being avoided when different tracks are used.

(1) Click on the <Acquire> and <Config> buttons from the LSM 510 tool bar. The **Configuration Control** menu appears on the screen.

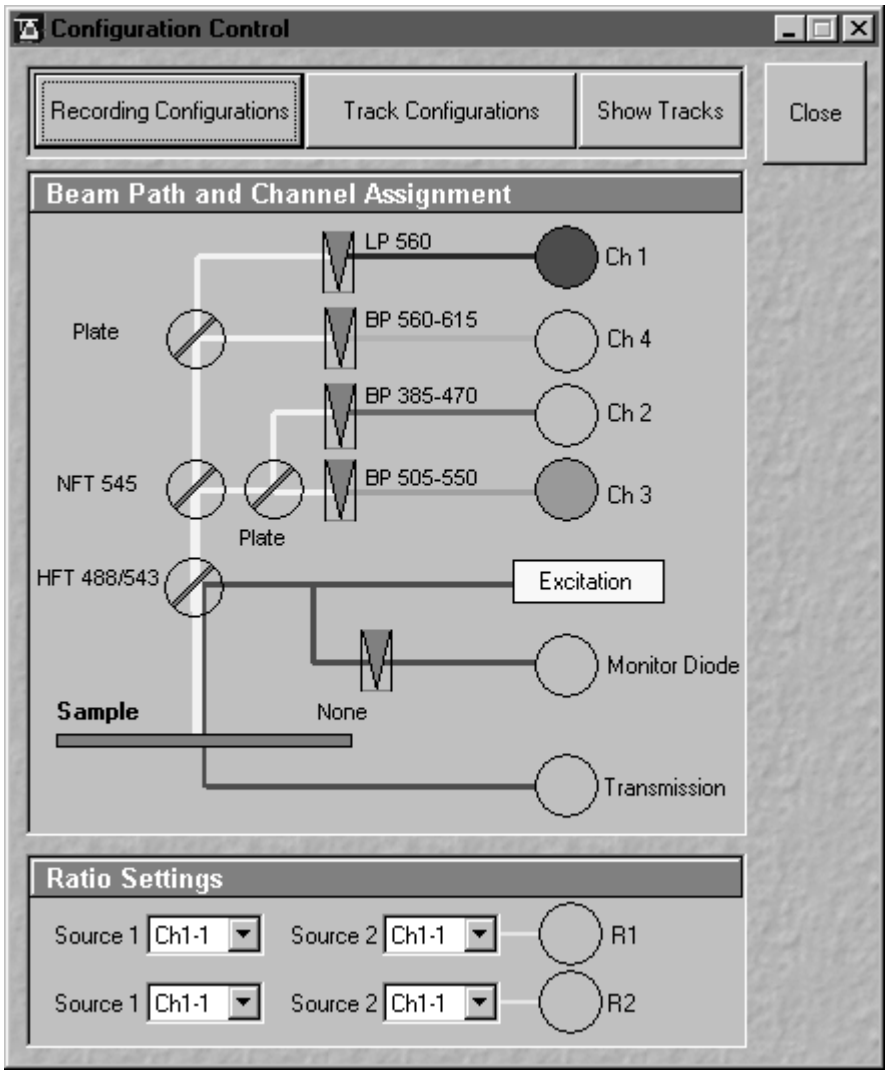


Fig. 4-43

(2) Click on the <Show Tracks> button on the right side of the window.

Within the "Configuration Control" window, the additional **List of Tracks** panel appears. The following functions are available in this dialog box:

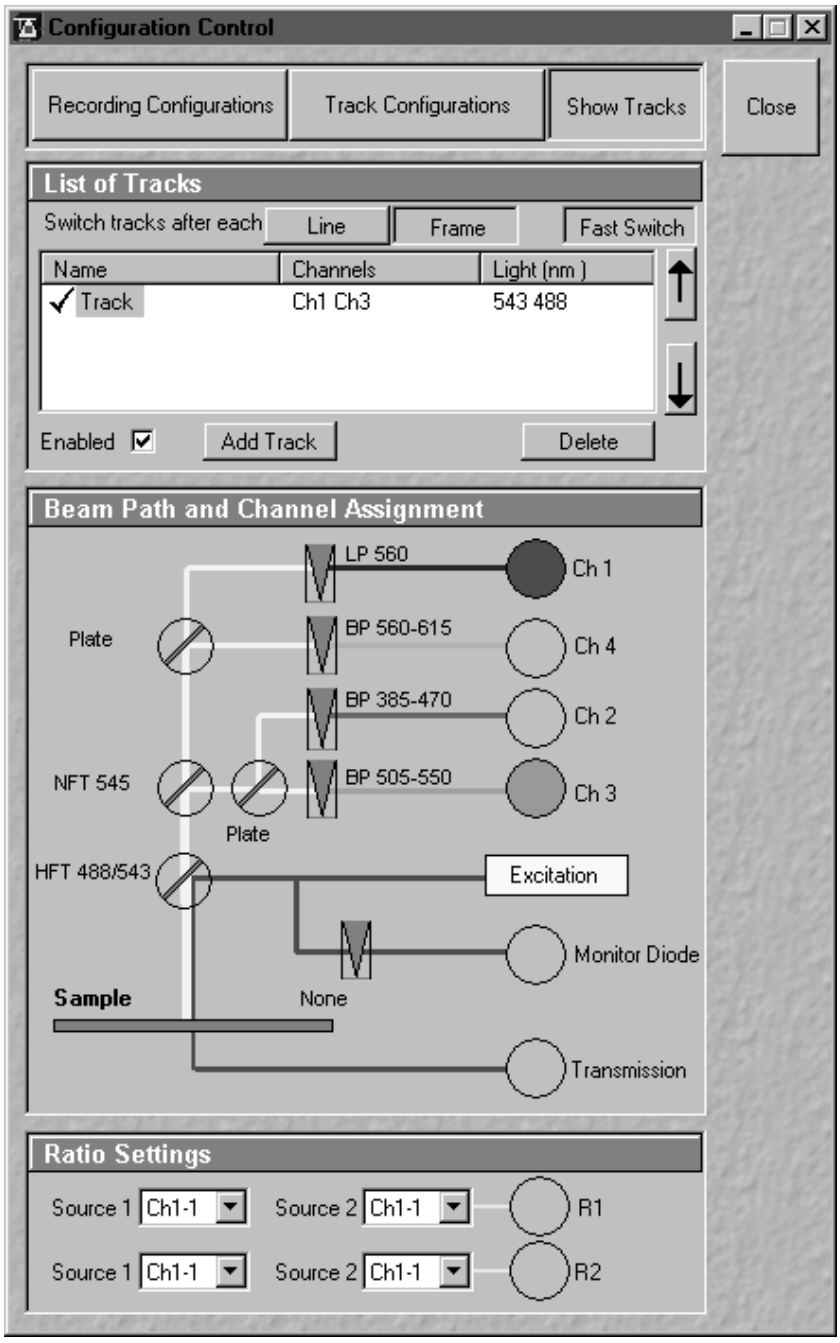


Fig. 4-44

<Add Track> button	An additional track is added to the configuration list. The maximum of four tracks can be added.
<Delete> button	The track previously marked in the **List of Tracks** panel in the **Name** column is deleted.
<Enabled> button	The track previously marked in the **List of Tracks** panel in the **Name** column is activated or deactivated. A tick ☑ in the button and also in the **List of Tracks** indicates that the relevant track is activated.

When adding new tracks, the following sequence should be followed:

(1) Add a track by clicking on the <Add Track> button.

(2) Determine the configuration of the track in the **Beam Path and Channel Assignment** panel or select an existing one from **Track Configurations** and activate it via the <Apply> button.

(3) Store the name of a configuration defined via the <Track Configurations> button via <Store> and use <Apply> for activation. The new track will then be displayed in the **List of Tracks**.

(4) Add the next track via the <Add Track> button and then configure and store it again.

The following different functions are available for storage:

<Recording Configurations> button	Stores the complete configuration data and not just the track configuration.
<Track Configurations> button	Only the configuration of the selected track is stored.

☞ When the **Recording Configurations** function is used, additional tracks cannot be configured. Only the entire configuration can be changed.

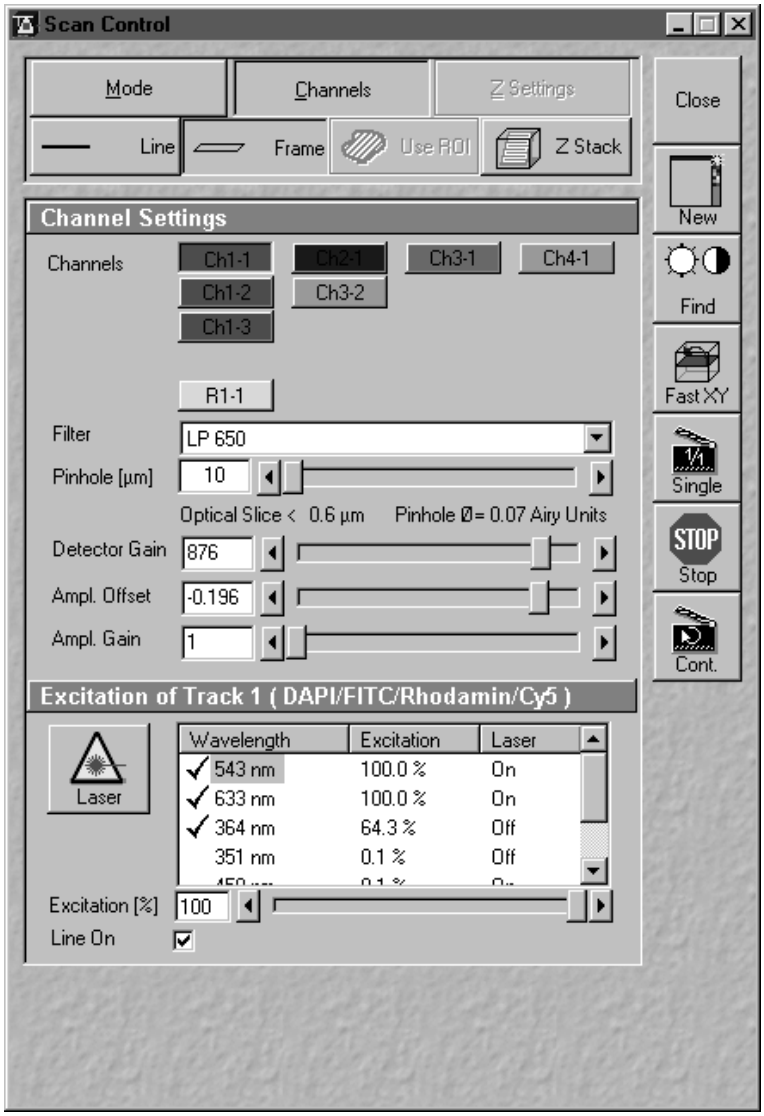


Fig. 4-45

<Frame> button	The entire area is scanned, and then the parameters are reset.
<Fast Switch> button	The scanning procedure can be made faster. Only the Acousto-Optical Tunable Filters (AOTF) are switched, and no other hardware components.
<Line> button	The new parameters are called up after every scanned line.

☞ When <Line> is selected, the same rules apply as for <Fast switch>.

Clicking the <Scan> button in the LSM 510 tool bar and the <Channels> button in the <Scan Control> window allows viewing of the channel configuration set before.

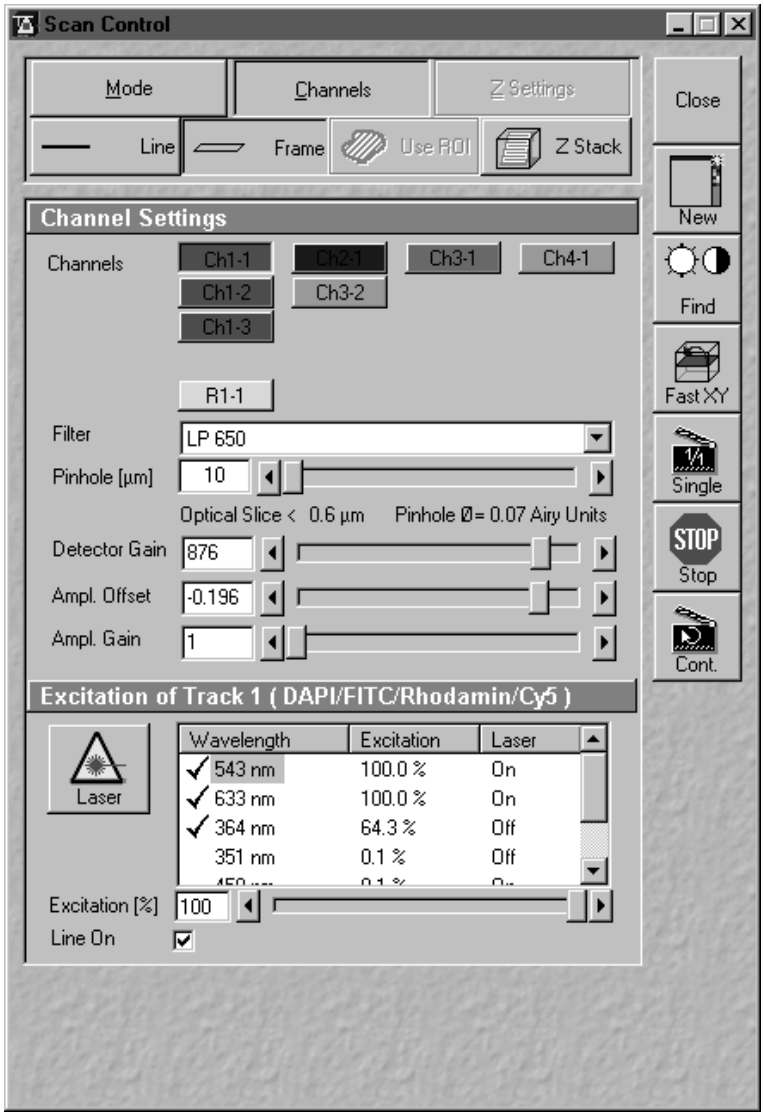


Fig. 4-46

The following scan image shows the result with two defined tracks plus the ratio track and the overlay.

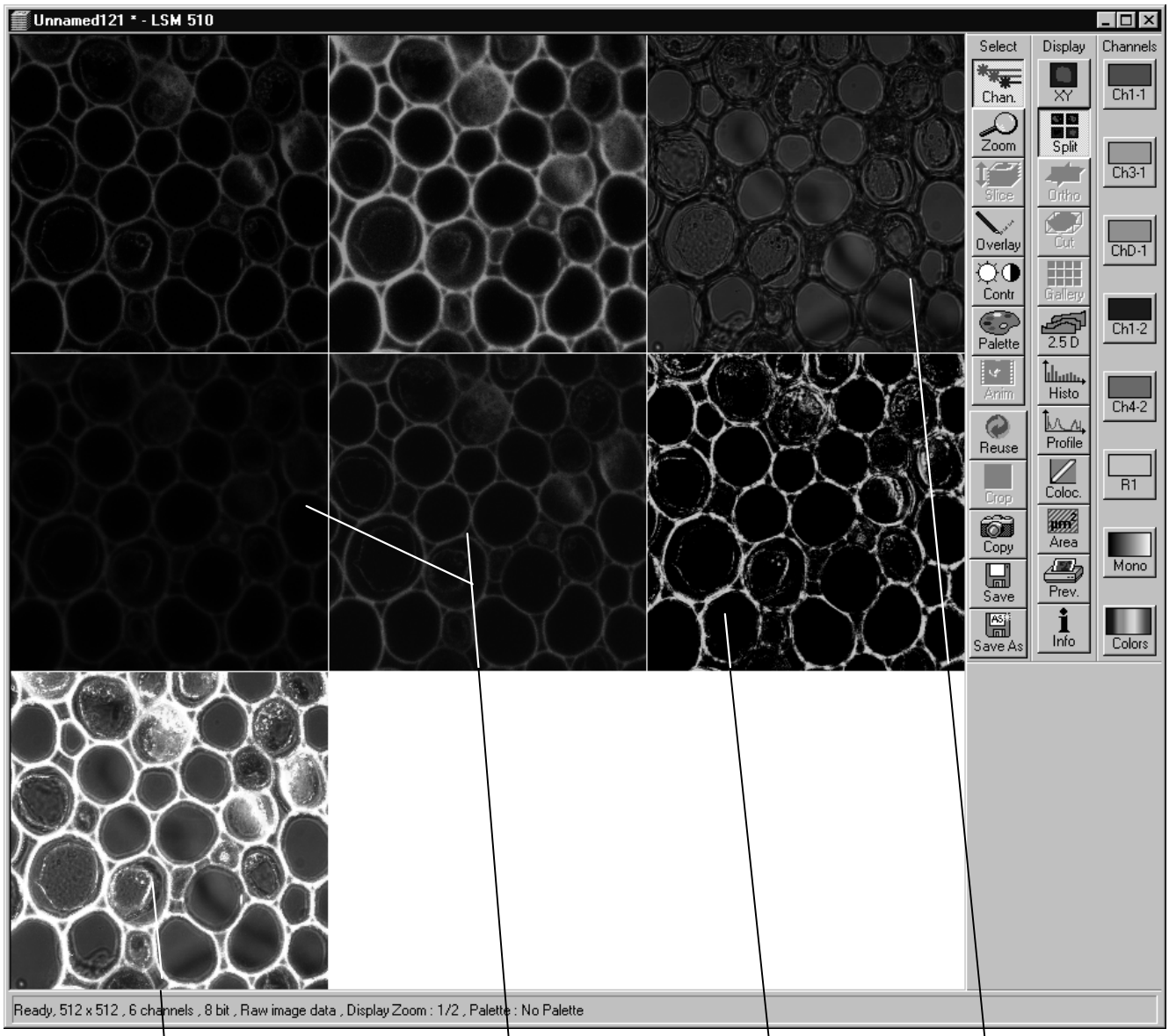


Fig. 4-47

Overlay

2. Track:
-Ch 1-2
-Ch 4-2

Ratio-Track:
-R 1-1

1. Track:
-Ch 1-1
-Ch 3-1
-Ch D-1

The **Ratio Settings** box in the "Configuration Control" window makes it possible to determine from which of the two channels a ratio channel is created. The interrelation between the channels can be determined through three formulas available for selection.

Clicking on the <Scan> button in the LSM 510 tool bar and the <Channels> button in the "Scan Control" window will display the defined channels and the ratio channel in the **Channel Settings** panel.

Clicking on the <R1-1> button for the ratio channel will open the following window:

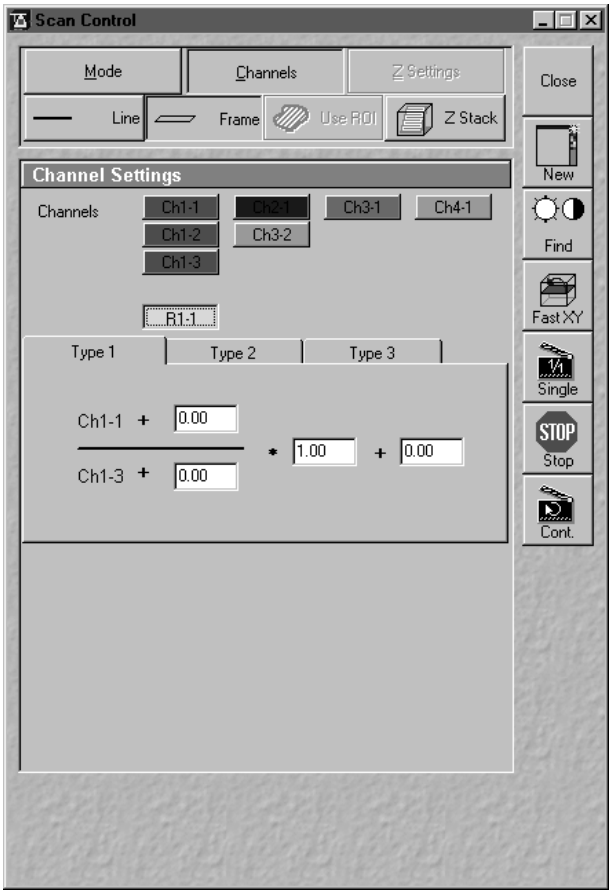


Fig. 4-48

Three formulas are available for the linking of the two channels. Selecting the formula via the <Type1>, <Type 2> and <Type 3> buttons will automatically store the formula and thus define the ratio channel.

Note:

- **Recording Configuration** is not applicable in the case of **Add Track**
- If a **Track** is deactivated via **Ratio Channel**, the ratio channel is deleted.
- Optimization via the **Find** function is possible only for each individual **Track**.

Laser attenuation

- On the **Beam Path and Channel Assignment** panel, move the cursor to the <Excitation> button.
- Once the cursor has changed into a hand symbol, click on the button.
  - This opens a drop-down list of all available lasers with their wavelengths and their usable AOTF attenuation.
- To select the desired laser, click on the respective line in the list.
  - The selected laser will be highlighted in blue.
- Use the Excitation [%] slider to set the laser power (recommendation: start at 50 %).
  - The transmittance of the Acousto-Optical Tunable Filter (AOTF) changes accordingly.

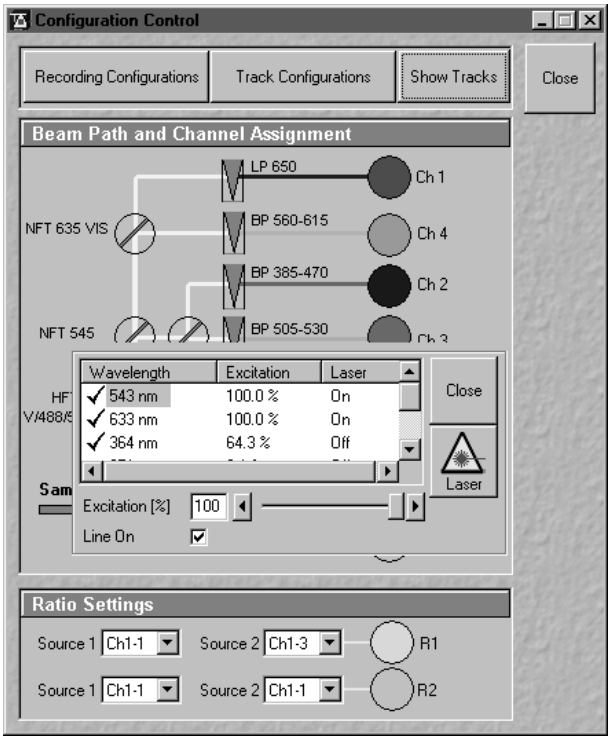


Fig. 4-49

☞ If you deactivate "Line On", the laser wavelengths for Enterprise and argon lasers are deselected by means of the AOTF, i.e. these lasers change into standby status.

If you interrupt your work with the LSM for a break, it is recommended not to switch the Enterprise and argon lasers off by hardware action, but to put them into standby status as described.

Scanning operation is not possible in the standby status.

Operate the Excitation slider as follows:

- Click on the slider and shift it while keeping the left mouse button pressed.
- Keep the <Shift> key pressed and click on the ◀ or ▶ arrow button to vary the power in steps of ten.
- Keep the <Caps Lock> key pressed and click on the ◀ or ▶ arrow button to vary the power in steps of one.
- Keep the <Ctrl> key pressed and click on the ◀ or ▶ arrow button to vary the power in steps of one tenth.

- Use the AOTF to set the laser intensity not completely to zero, but only to 0.1 %.

This allows you to adapt the laser intensity very sensitively to the job.

☞
- Click on <Line On>.
  - This activates the selected laser power for use. This is indicated by the laser status line displaying "On".

  By clicking on the <Excitation> button you can check at any time which lasers are available for active operation.

- Click on the <Close> button to conclude the settings procedure.

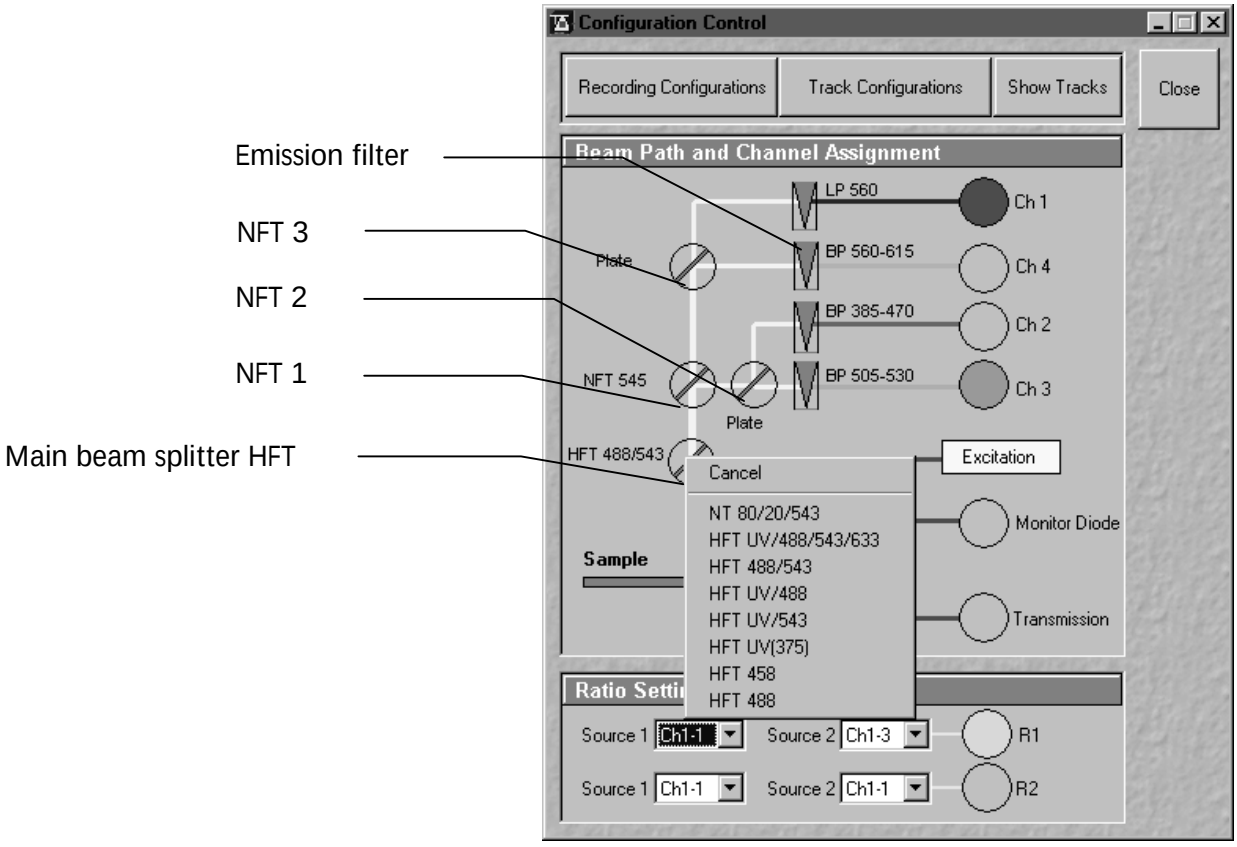


Fig. 4-50

Beam path - Main beam splitter

- On the **Beam Path and Channel Assignment** panel, move the cursor to the symbol of the main beam splitter HFT ⊘.
- Click on the symbol once the cursor has changed into a hand.
  - This opens a drop-down list of all beam splitters available.
- To select a beam splitter, click on the respective line of the list.
  - The selected beam splitter moves into the beam path.

Photomultipliers

- On the **Beam Path and Channel Assignment** panel, move the cursor on one of the channel symbols, e.g. Ch1.
- Click on the symbol once the cursor has changed into a hand.
  - This opens a color selection table "Channel Color Selection" on the **Beam Path and Channel Assignment** panel.
- Click on the desired color spot.
  - This activates photomultiplier PMT1.
- Proceed in the same way for the other PMTs.

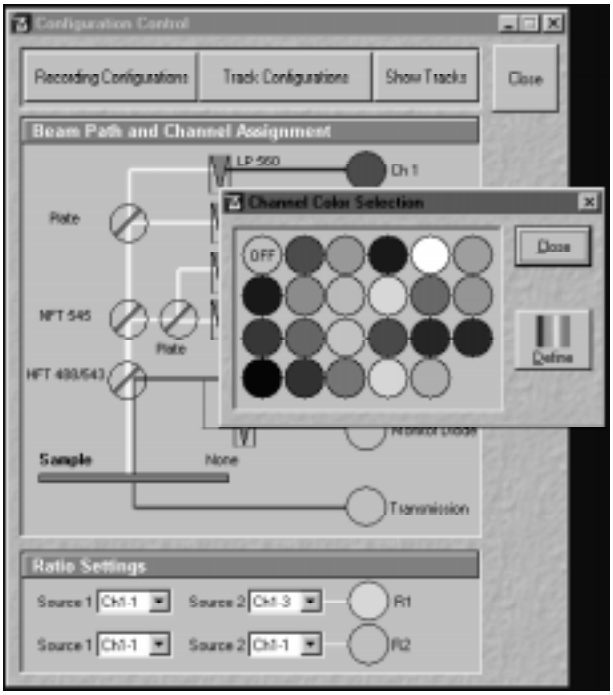


Fig. 4-51

Clicking on the OFF spot results in deactivation of the corresponding channel.

Further colors for the corresponding channel can be produced as follows:

Clicking on the <Define> button will open a further "Channel Colors" window. Via a reticule in the "Define Color" dialog box, any desired color can be produced. Clicking on the <Add> button allows the color to be used for further channel coloring.

- Choose the desired color with the reticule (the reticule is in the left corner at the bottom of the color range).
- Define the brightness by use of the scroll bar.
- Use the <Add> button to add the color to the color range or remove the marked color using the <Remove> button.

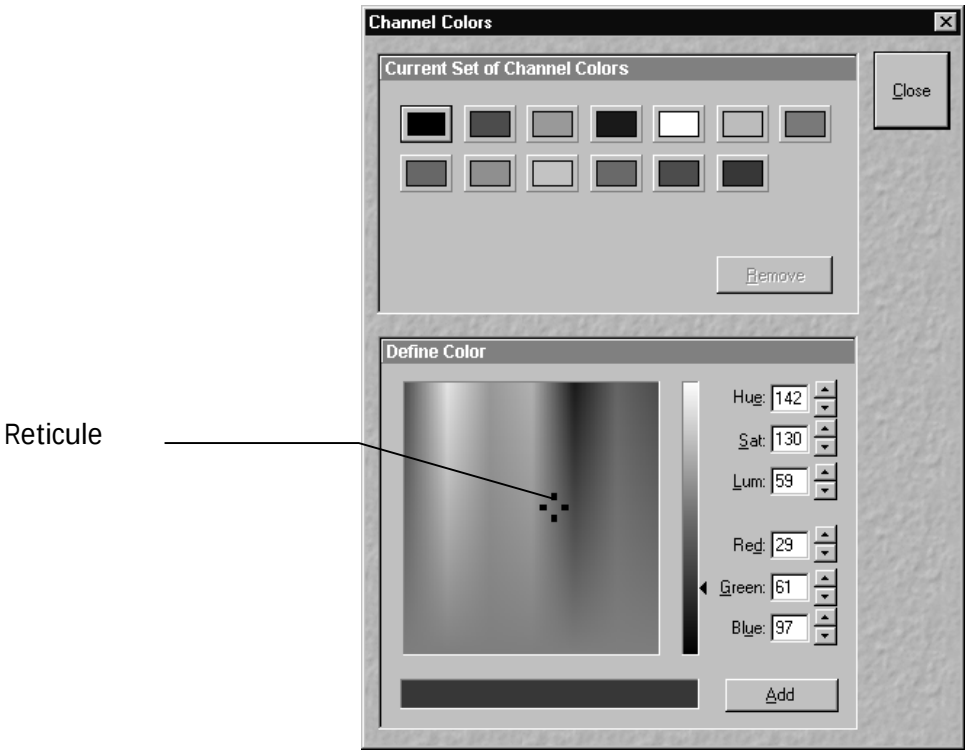


Fig. 4-52

Emission filter

- On the **Beam Path and Channel Assignment** panel, move the cursor to the emission filter symbol.
- Click on the symbol once the cursor has changed into a hand.
  - This opens a drop-down list of all available emission filters (e.g. BP for band pass, or LP for long pass) with their wavelengths.
- To select an emission filter, click on the respective line in the list.
  - The emission filter selected moves into the beam path in front of the PMT.

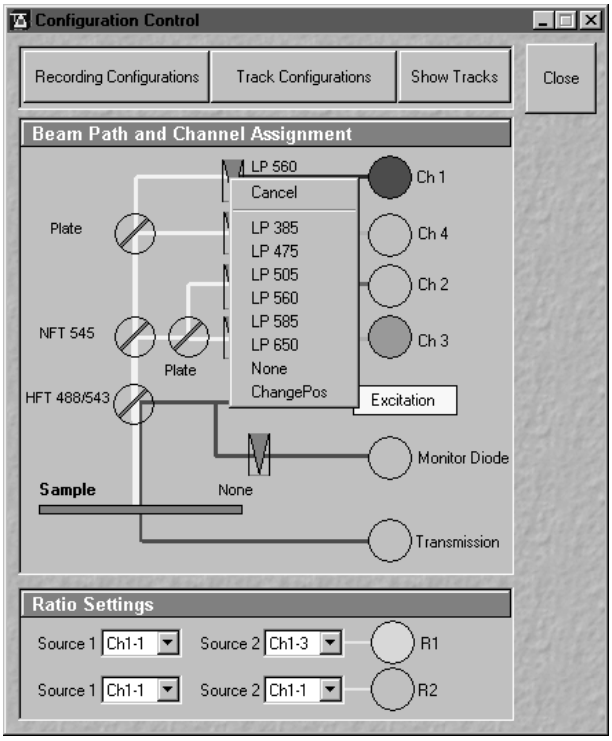


Fig. 4-53

- Depending on the application, it may be necessary to insert additional mirrors, dichroic beam splitters or neutral glass filters between the main dichroic splitter HFT and the photomultiplier PMT. To select these components, click on the respective symbols.
- Click on the <Close> button to quit the "Configuration" window.

This completes the configuration for the scanning process described in section 4.5.4.

☞ The symbols for the transmitted-light PMT (Transmission) and Monitor Diode can be activated in the same way as the PMTs of channels 1 to 4.

When changing from NFT 1, intelligent adaptation of NFT 3 is performed automatically.

4.5.4 Scanning modes

Taking a simple configuration as an example, the sections below describe the procedure for acquiring a single scanned image in single-channel presentation, using fluorescence and confocal imaging.

Requirements

Axioplan 2 MOT

- Push in the silver slider (4-37/**8**) on the microscope tube as far as it will go.
- Activate the "Axioplan Control" window by mouse click, or, if the window was closed before, call it up again by clicking on the <Micro> button.
- Continue as described in sections 4.5.2.1 and 4.5.2.2.
- The specimen must have been positioned and focused by conventional eyepiece observation in fluorescent or transmitted light.

Axiovert 100 M BP

- Perform the settings in the same way as described for the Axioplan. Make sure that the sideport slider (4-39/**2**) and VIS slider (4-39/**3**) are in the correct positions.

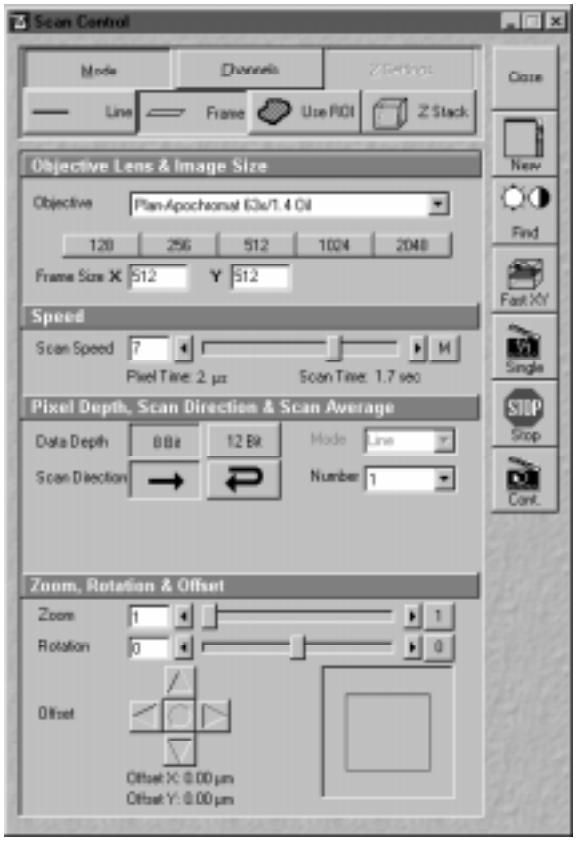


Fig. 4-54

Scanning

- Click on the <Scan> button in the main menu.
  - This opens the "Scan Control" window, in which all necessary scanning parameters can be set.

The first selection to be made is whether you want to perform a

- Line scan
- Frame scan
- Use ROI or
- Z stack.

This selection will be made via the <Mode> button, followed by activation of the desired scan mode.

The following description is limited to the Frame and Z modes. For "Line Scan", proceed in the same way.

4.5.4.1 Frame

- Before starting, click on the <Mode> button on the upper toolbar.
- Click on <Frame> on the lower toolbar.
  - The toolbar changes to correspond to the Frame selection.
- On the **Objective Lens & Image Size** panel, select the following parameters:
  - Objective (the objective currently in the beam path is indicated)
  - Frame Size (on the **Scan Size** panel, select the size of the scanning frame by clicking on the respective button. It is also possible to directly enter the frame dimensions in pixels).

    Recommended setting to start with:
    512x512 pixels.
- On the **Speed** panel, select the following parameter:
  - Use the Scan Speed slider to set the scanning speed needed.

    Recommended: 7 for the first scan.

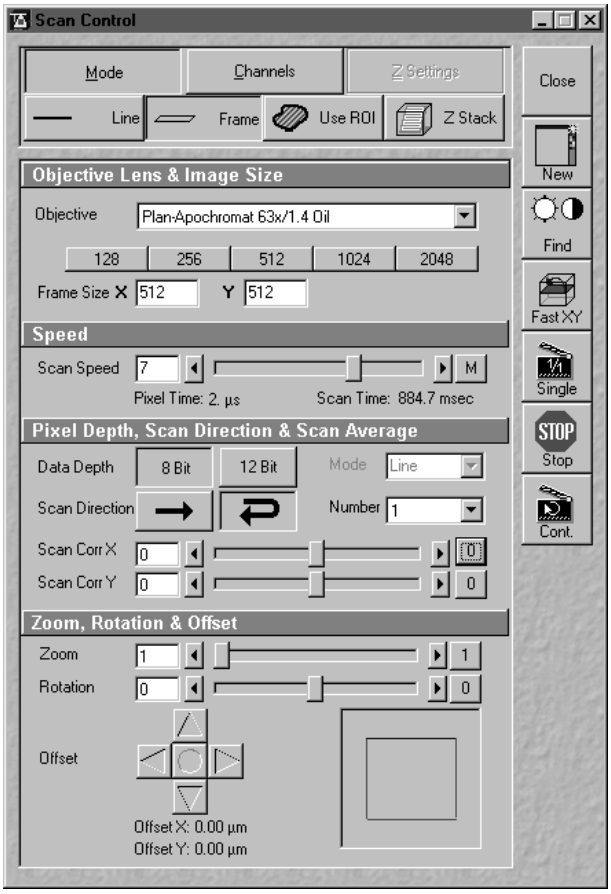


Fig. 4-55

☞ Speed 10 = fast xy, only for fast image acquisition during parameter setup
Pixel time and scan time will be shown.

- On the **Pixel Depth, Scan Direction & Scan Average** panel, select the following parameters:
  - Pixel Depth (intensity steps).
  - Scan Direction (uni- or bidirectional scan).

When clicking on the button for bidirectional scanning, a possibility to correct the pixel shift is offered. The correction possibly required depends on the scanning speed.

- Select the number of image averaging (can be selected from 1 to 8 under "**Number**") to improve the quality of noise-impaired images.

Line Scan is only possible in the unidirectional mode.

☞ The greater the number of averages selected, the better the image quality will be; the scanning time will be prolonged accordingly.

- On the **Zoom, Rotation & Offset** panel, use the slider to set the desired zoom factor between 1 and 8. This adjustment is infinite.
  - The scanning frame thus defined is shown by a red outline.

    The scanning frame can be rotated and translated at liberty.

    Recommended setting to start with: Zoom 1, Rotation 0

Please bear in mind that a reference scale which might be included in the image during the scanning procedure will only be activated after the end of the scanning procedure if you change the zoom factor

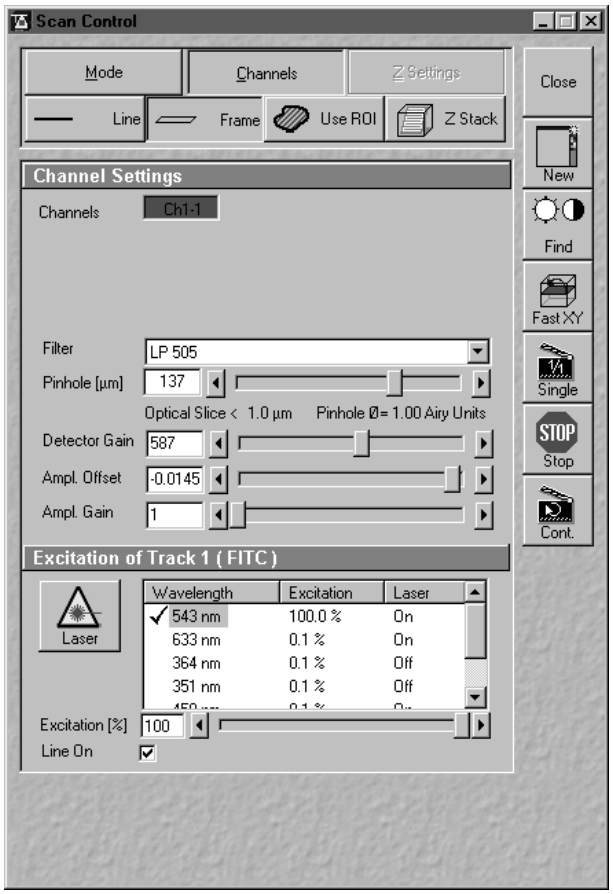


Fig. 4-56

- Click on <Channels> in the upper toolbar.
  - This opens the new dialog boxes **Channel Settings** and **Excitation of Track,** showing the previously set parameters, which can, however, be edited.
- Optimize the image quality by varying the pinhole aperture:
  - A small pinhole diameter will increase the depth of focus, but reduce the light intensity received by the PMT.
  - When you vary the pinhole diameter, an Optical Slice value is displayed. For optimum depth resolution, Airy values should be small, but not below 1.0 to keep the intensity loss within a reasonable limit.

☞ The parameters

- Detector Gain
- Ampl. Offset
- Ampl. Gain

are described in sections 4.7.1 in the context of image optimization.

- In the **Excitation of Track** dialog box you can select other lasers and vary laser intensities.

4.5.4.2 Z mode

This mode allows you to create a stack of images at different Z levels (slices).

- In the "Scan Control" window on the <Z Stack> button.
  - This opens the **Z Settings** panel in the "Scan Control" window.

The scanning range in Z can be determined as an option via the following functions:

- Z Sectioning (input of numerical values)
- Cutline and Range (in monitor dialog with the specimen)
- Mark First/Last (in monitor dialog with the specimen)

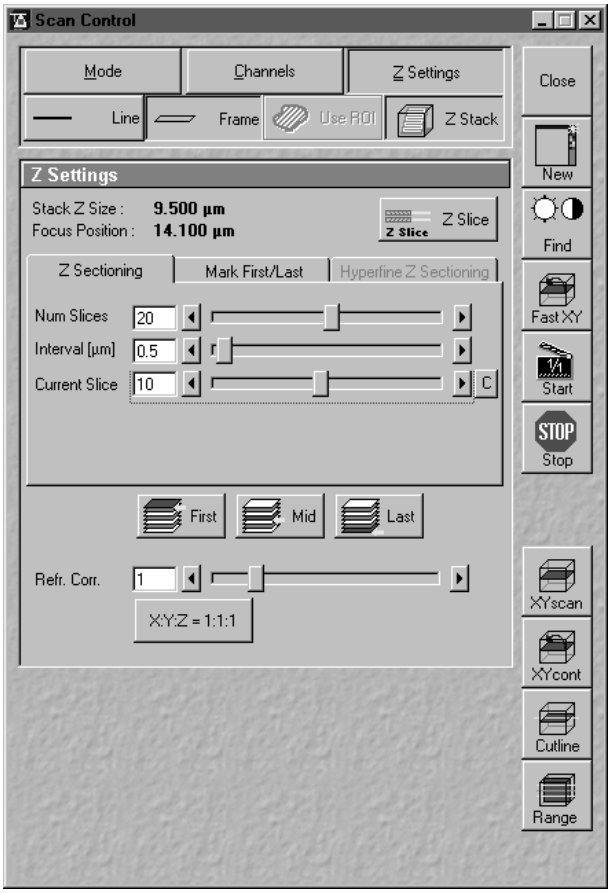


Fig. 4-57

Z Sectioning

- Activate the Z Sectioning register
- Use the **Num Slices** slider to select the number of slices in the stack.
- Use the **Interval [µm]** slider to select the Z interval between successive slices.
- Use the **Current Slice** slider to select the center of the stack.
- Click on the <Start> button to trigger Z Sectioning.

Optimization of intervals, number of slices and pinhole diameter

- Click on the <Z Slice> button to determine the optimum interval

The **Optical Slice** dialog box (see Fig 4-59) will appear, from which the optimum interval (depending on the used objective and pinhole diameter) can be seen and made available to the active mode.

Clicking on the <Optimal Pinhole Diameter> button also enables the optimum pinhole diameter to be set.

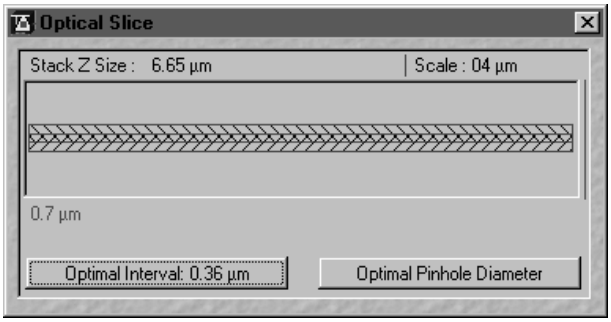


Fig. 4-58

The **Optical Slice** dialog box displays the following information:

Black:

Stack Z Size (µm) = intervals x (number of slices - 1)
Optimal Interval = depending on the objective used and the pinhole diameter setting

Red and other colors:

Presentation of the actual data set by the operator helps to optimize stack creation.

- Click on the <XY scan> button.
  - This opens an extra window showing the scanned image.

☞ The scanned image can be optimized by modifying the relevant parameters with the XY Cont. function (continuous scanning, section 4.9.2).

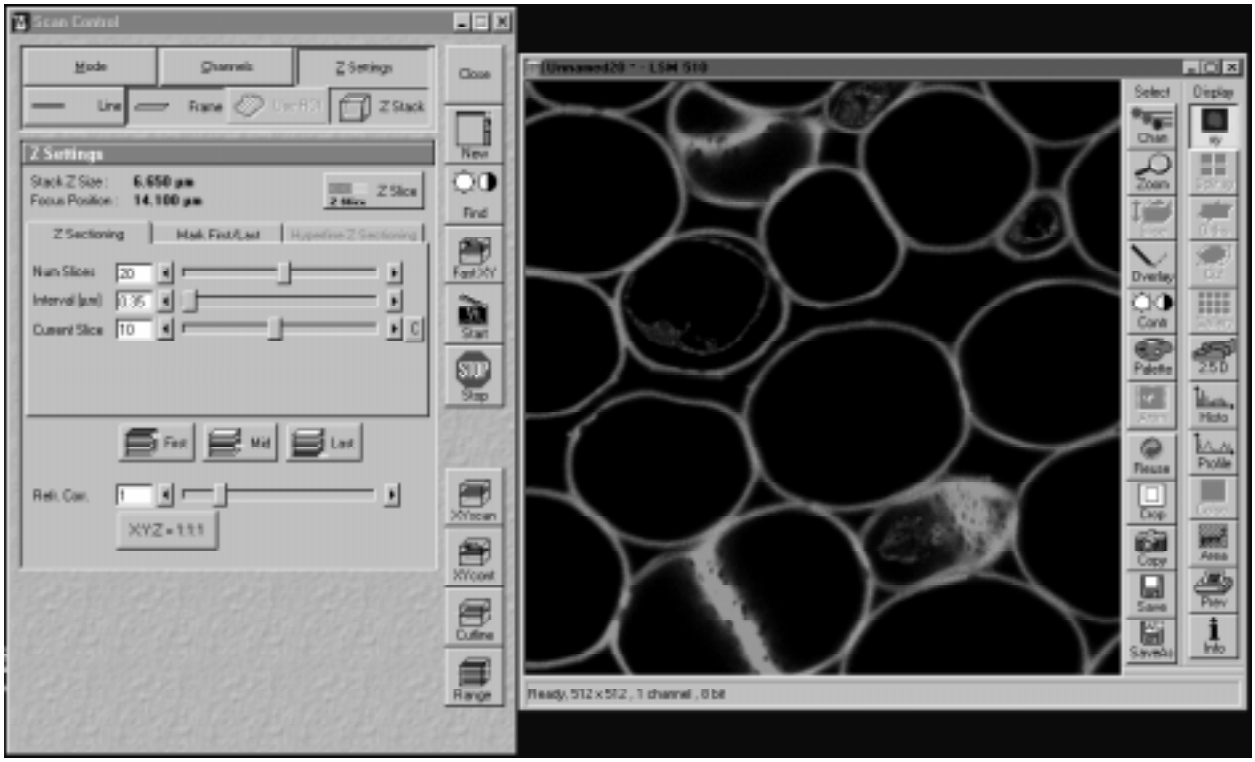


Fig. 4-59

Cutline and Range

For a useful evaluation of the z stack you must proceed as follows:

- Click on the <Cutline> button.
  - The image window shows the course of the red cutline.

Clicking on the cutline allows you to remove the line to the position where the specimen should be cut.

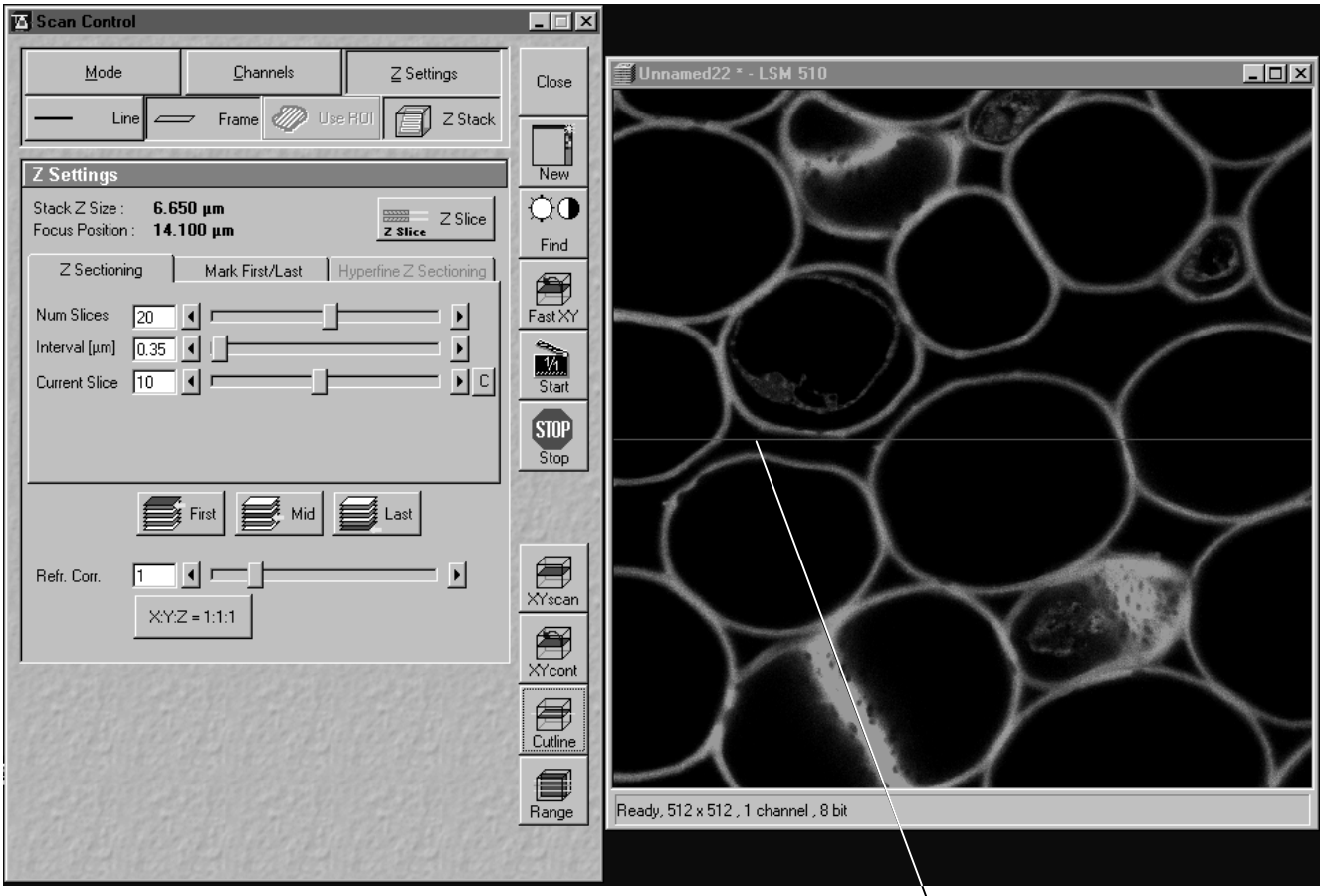


Fig. 4-60

- Click on the <Range> button.
  - The image window marks the range to be scanned by red outlines.

☞ Not only the current slice (green cutline) but also the red range lines can be removed by moving the cursor in the image window.

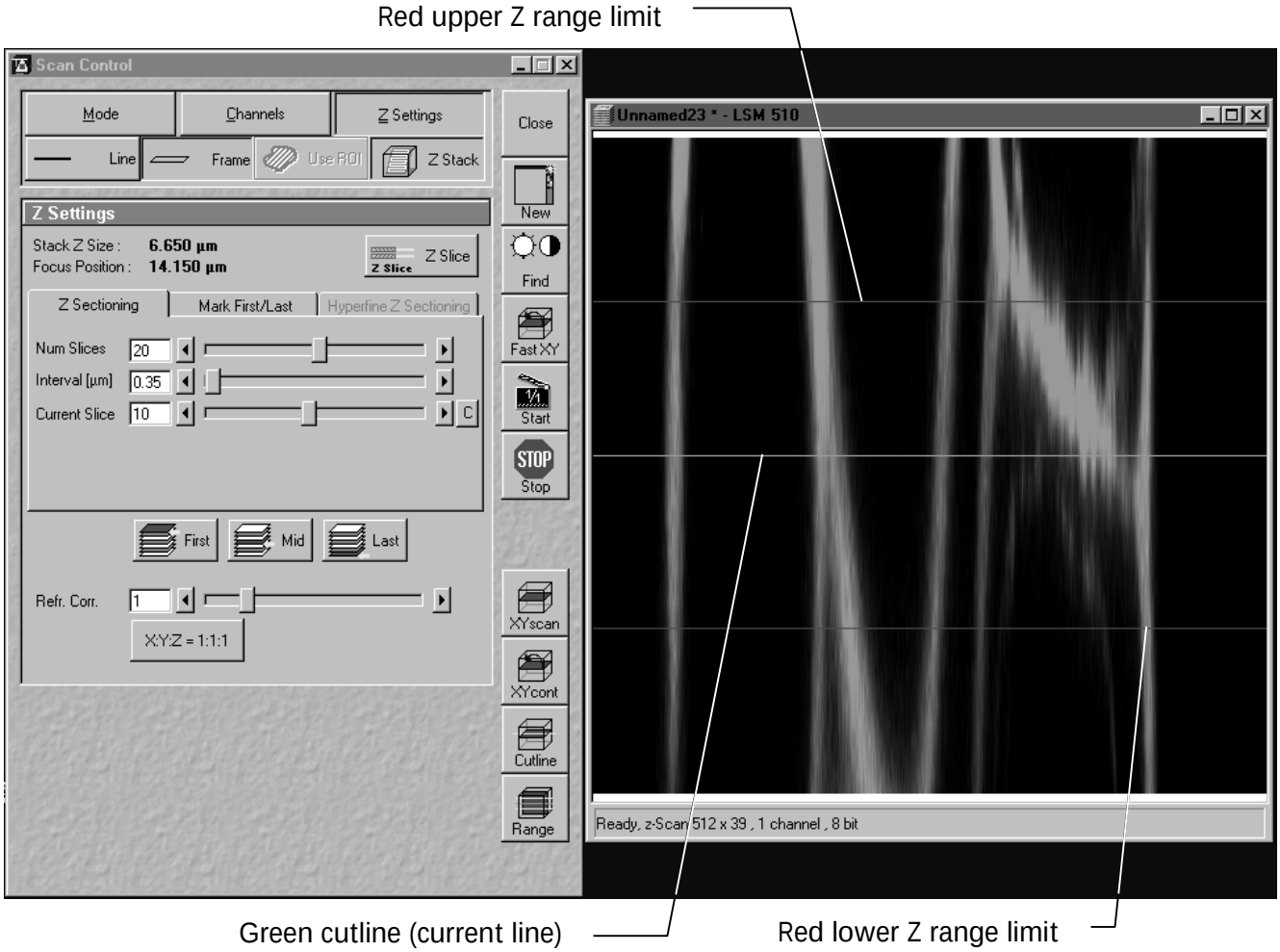


Fig. 4-61

Mark First/Last

Optimum stack size can be obtained by manual focusing during scan movement to the level where the stack should start. Proceed as follows

- Open the **Mark First/Last** register.
- Start the scanning process by clicking the <XYcont> button.
- Use focusing drive to set the focus position to the required Z-plane and press **Mark First** button.
- Then focus on the opposite Z-plane and press **Mark Last** button.

The XY-Cont Scan must be finished via <Stop> before acquisition of Z Stacks can be startet via <Start>.

The <First>, <Mid> and <Last> buttons allow a fast overview of the first, middle and last scanning step.

- Click on the <Stop> button.
- Click on the <Start> button.
  - Scanning process for stack recording will be started.

Before the <Start> button is pressed, the following settings must be made and checked:

- Depending on the immersion medium used, the suitable correction value must be selected under **Refr. Corr**. This correction value is the quotient of n/n', n = refractive index of the sample and n' = refractive index of immersion medium.

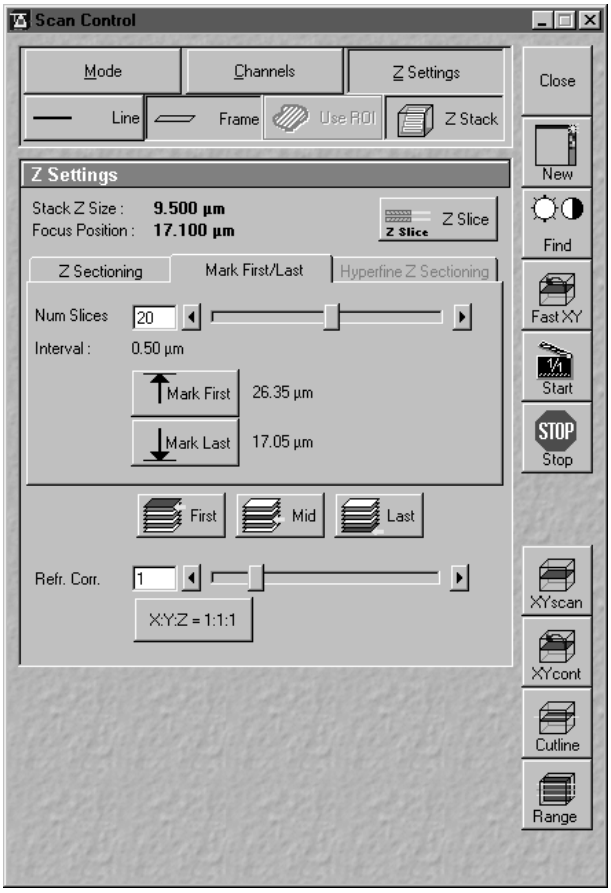


Fig. 4-62

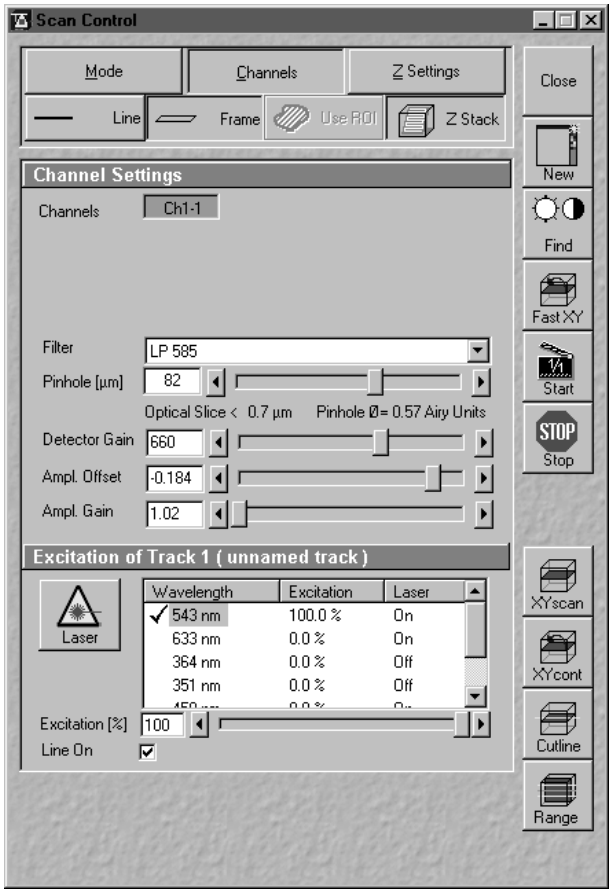


Fig. 4-63

When the <Z Stack> and <Mode> buttons are activated, objective can be changed, number of pixel and scan speed can be set; desired average and number of gray shade can be chosen as well as uni- or bidirectional scan; and the desired zoom, rotation and offset can be set.

When <Z Stack> and <Channels> are activated, picture can be optimized during scan.

4.5.4.3 ROI function / Region of Interest

A scan image allows certain areas (ROIs) to be defined. Only these areas of interest will be scanned. The laser beam will be switched in only in these areas via AOTF.

(1) Click on the <Acquire> and <Edit ROI> buttons from the LSM 510 tool bar. The "Edit ROI" window appears on the screen.

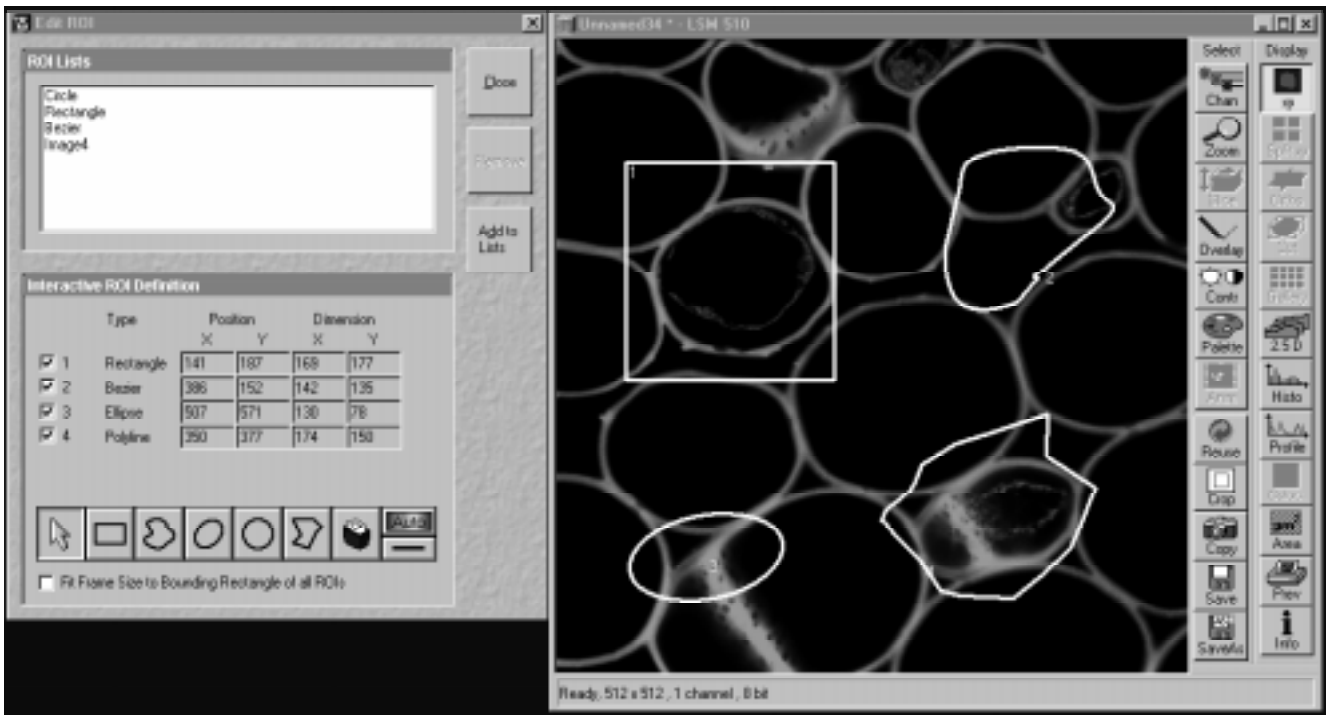


Fig. 4-64

(2) In the tool bar of the **Interactive ROI Definition** panel, click on the symbol of the area you want to use to mark the region of interest in the scanning image. Five different area symbols are available in the form of buttons.

(3) Click on the marking area and keep the mouse button pressed to drag the area into the region of interest in the scanning image. The marking area will be numbered automatically and entered in the **Interactive ROI Definition** panel with its position and dimension parameters and the appropriate number.

(4) The dragged marking area is marked by clicking on its outline; its size can be increased or reduced by clicking on the marking points. Clicking on the area edge beside the marking points allows repositioning of the area on the scanning image.

(5) If you have framed all the required ROIs in accordance with steps 2 to 4, you can store these ROIs under any required name via the <Add to Lists> button.

(6) The "Add ROI List" window will appear. Enter any required name to store the ROIs and click on the <OK> button.



Fig. 4-65

(7) This stored ROI configuration appears in the **ROI Lists** panel of the "Edit ROI" window.

The following functions are also available in the "Edit ROI" window:

<Close> button	The "Edit ROI" dialog box is closed.
<Remove> button	An entry marked in **ROI Lists** (stored ROI configuration) is deleted.
<Waste-paper basket> button	All the ROIs dragged to the scanning image are deleted. If an area outline was marked before, this area is now deleted in the scanning image.
<Auto> button	A defined color from the list of colors can be assigned to the ROIs. In the **Auto** position, the outlines of the dragged ROIs are automatically colored differently.
<—> button	This button allows you to determine the line thickness of the area outline.
<☑> button	Clicking on this button in the **Edit** box allows a ROI to be deactivated. The tick disappears from the button, as does the relevant marked area from the scanning image. Clicking on the button again will reactivate the ROI.

The **Use ROI** status display in the "Scan Control" window shows whether the ROI mode is activated or not. When **Edit ROI** is activated, the ROI mode is active automatically. If ROIs shall not be taken in consideration during scanning, this button must be used to deactivate the function prior to the scanning procedure.

The following is an example of a scanning procedure where the **ROI** function was activated.

Only the regions of interest defined before are visible in the scanning image, the other areas remain dark.

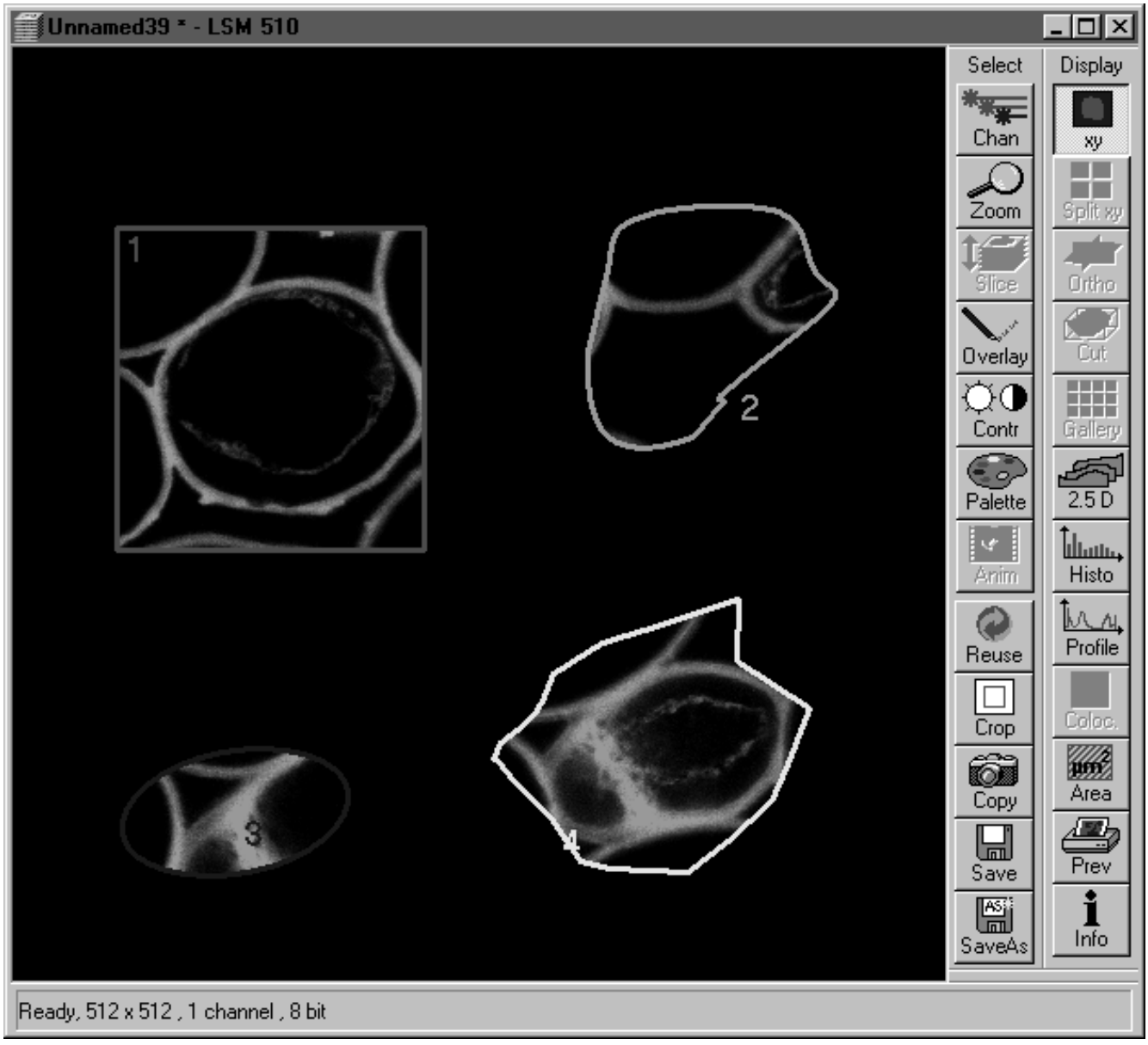


Fig. 4-66

4.5.4.4 Time Series function

The **Time Series** function offers the following options for the creation of a scanning image:

- Definition of break times between 0.1 ms and 10 hours.
- Determination of the number of steps from 1 to 10,000 for one scanning procedure.
- Setting of markers for a scanning procedure.
- Interruption of time control via break function, and start of the time function.
- Triggering of time series via:
  - numeric input
  - external trigger pulses
  - time

(1) Click on the <Acquire> and <TimeSeries> buttons in the LSM 510 tool bar. The "Time Series Control" window appears on the screen.

(2) Set the relevant parameters for time control in the **Start Series**, **End Series** and **Cycle Delay** boxes.

(3) Use the <Set 1> to <Set 7> buttons to set markers during the scanning procedure which will allow you to evaluate interesting scanning images later.

Note:

- "Time end" will finish time series even if you have created a program which would exceed the time end.
- Bleach times will be added.
- No break is possible during bleaching.
- If you want to integrate a bleaching procedure in a time series, start must be triggered via "Start B".
- If a time series is interrupted before its programmed end, the programmed number of images will be taken over in the database. However, only those images are stored which were created before interrupting the time series. This is due to the fact that the original image parameters are to be taken over via the **Reuse** function.

"Time Series Control" window enabling the entry of parameters for time series control.

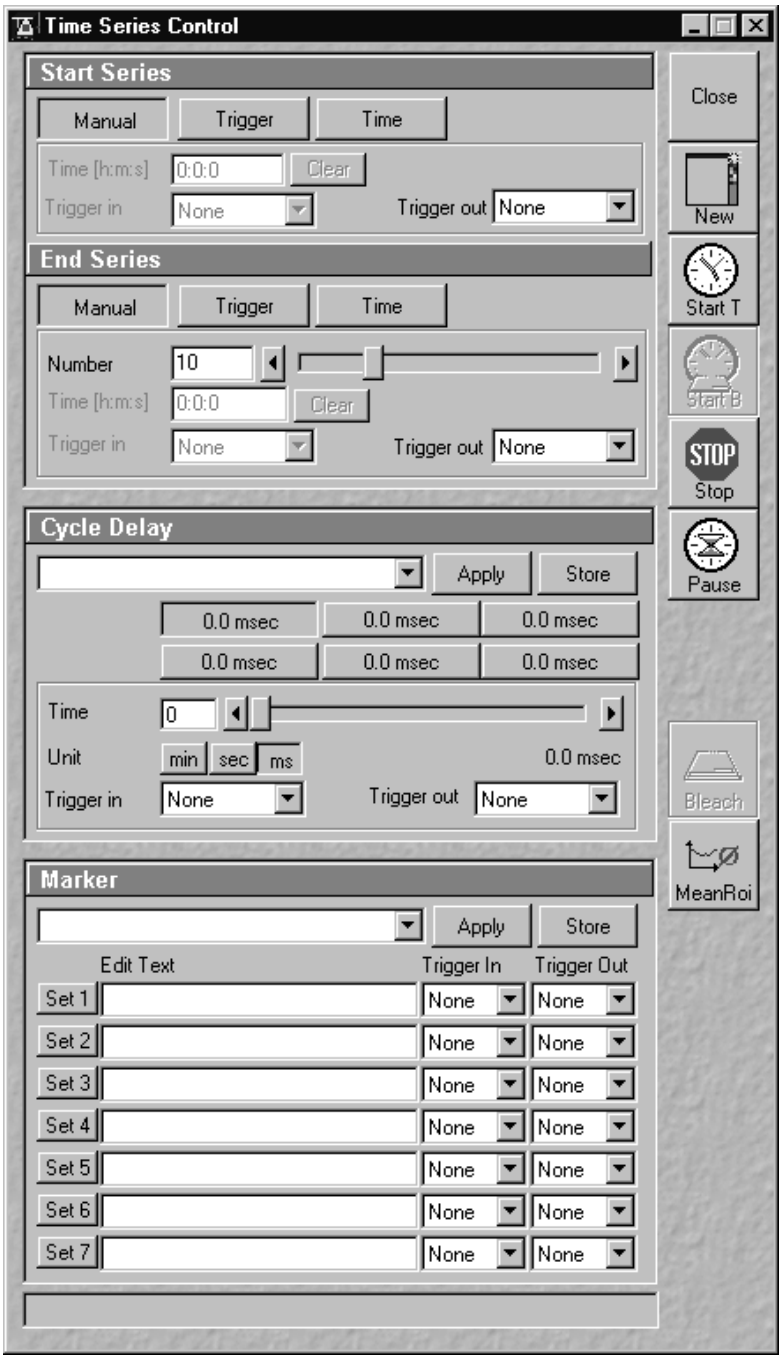


Fig. 4-67

The following example of a scanning image was taken using the **Time Series** function. Both the time and the markers set during the scanning procedure are projected in the image series in different colors.

If the cursor is moved to a marker position in the scanning image, the relevant information on the image detail is automatically provided in an additional window.

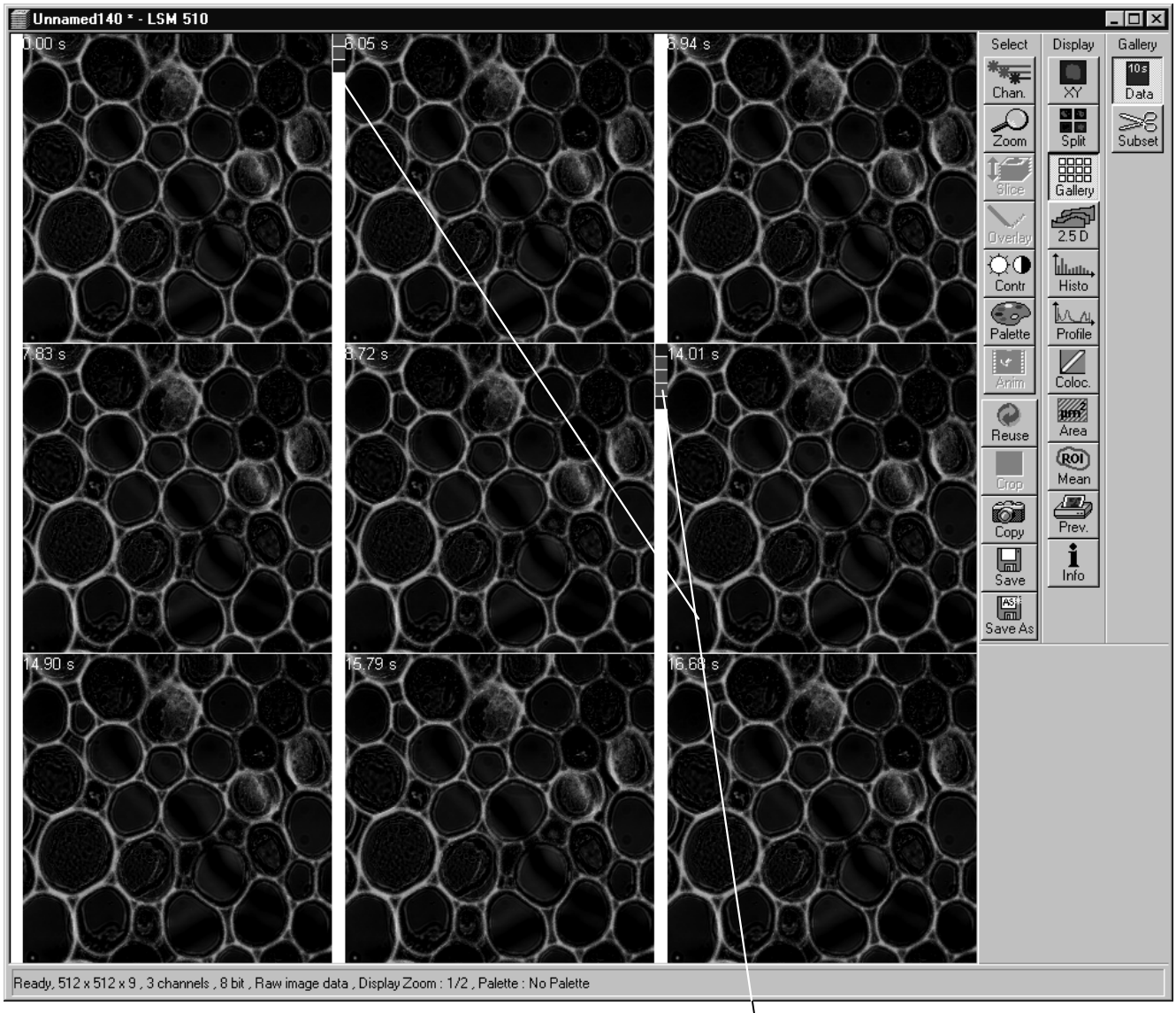


Fig. 4-68

4.5.4.5 Edit Bleach function

The use of this function permits the intense bleaching of a defined sample area.

To use the **Edit Bleach** function, proceed as follows:

- Click on the <Edit Bleach> button.
  - The "Bleach Control" window appears.
- The **Start Settings** panel allows you to determine when an how the bleaching process shall be triggered (only works in connection with time series).
- The **Bleach Parameter** allows you to determine how often the bleaching process shall be performed, and to select the area for bleaching in the scan image via the <Define Region> button.

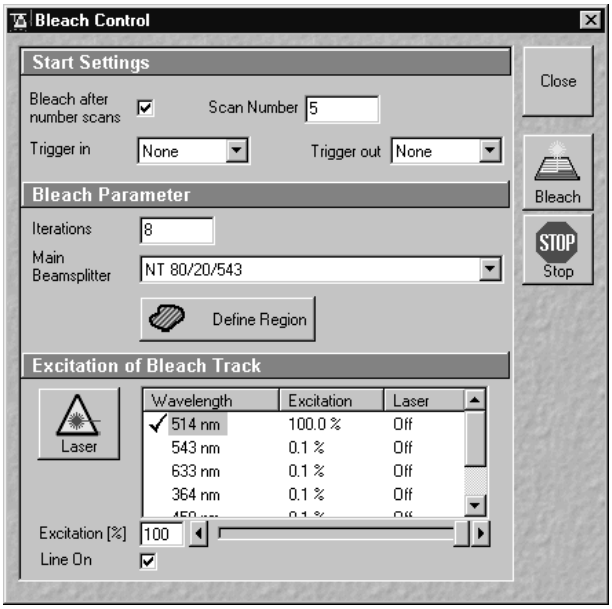


Fig. 4-69

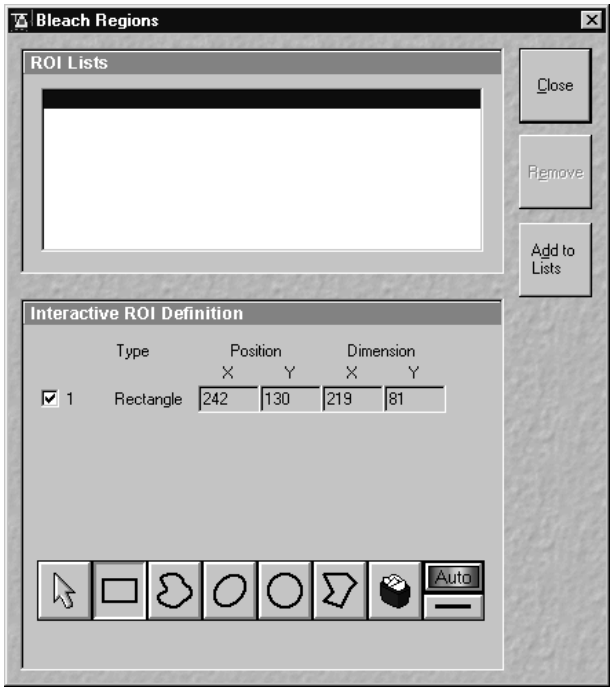


Fig. 4-70

- Click on the <Define Region> button.
  - The "Bleach Regions" window appears.
- Define the required bleach regions in the scan image.
- Select the required laser wavelength and its intensity under **Excitation of Bleach Track** in the "Bleach Control" window.
- The bleaching process will be started via the <Bleach> button. However, it is also possible to start the bleaching process via <Bleach> button in the "Time Series Control" window and to combine it with a time series.
- The bleaching process can be finished via <Stop> in the "Bleach Control" window.

☞ <Stop> does not only stop the bleaching process, but the entire scanning process.

4.6 Routine Mode

The Routine Mode of the LSM 510 software permits the fast and easy acquisition of scanning images by using time-tested **Standard Examination Methods** or by **User Defined Examination Methods**.

Standard Examination Methods are included in the LSM 510 software package and must only be activated once during the first application of the routine mode.

User Defined Examination Methods are methods which were already created and optimized in the Expert Mode. If **User Defined Examination Methods** are also to be used in the Routine Mode, they must be exported in the Routine Mode first. Apart from **User Defined Examination Methods,** stacks and time series can also be exported to the routine mode in addition to simple frames.



Fig. 4-71

4.6.1 Activate Standard Examination Methods in the Routine Mode

- Start the LSM program.
- Click on the <Start Routine Mode> button (Fig. 4-71) in the LSM 510 switchboard menu.
  - The "Routine Mode - Select Examination Method" window will appear.
- Click on the <Add method> button.
  - The "Select Method To Add" window appears on the screen.

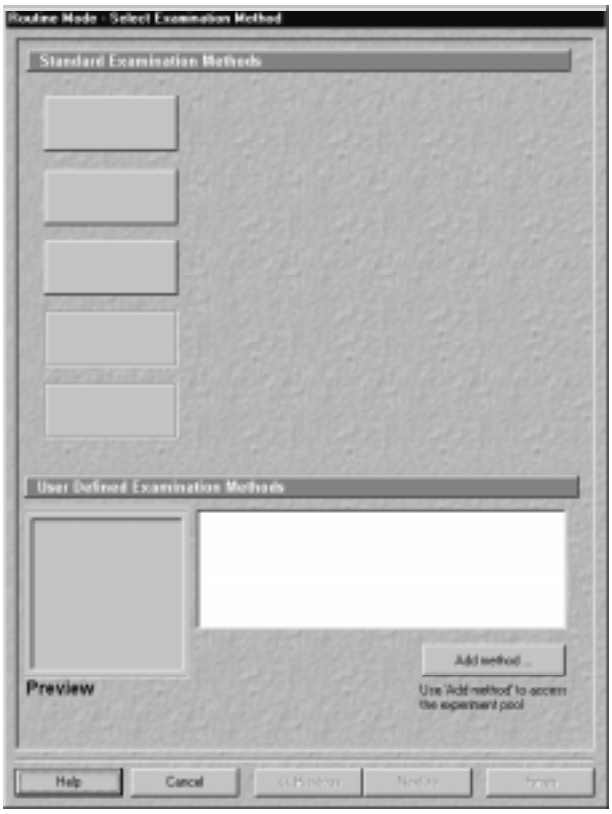


Fig. 4-72

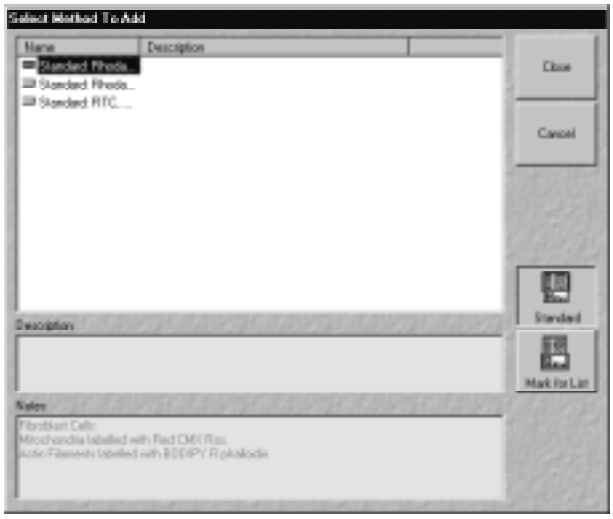


Fig. 4-73

- Select the method in the filed **Name** and click on the <Standard> button. An icon appears beside the name.
- Click on the <Close> button in the "Select Method To Add" window.
  - The standard examination methods are taken over and are then available in the LSM 510 switchboard menu.
  - The relevant labeling appears beside the buttons.

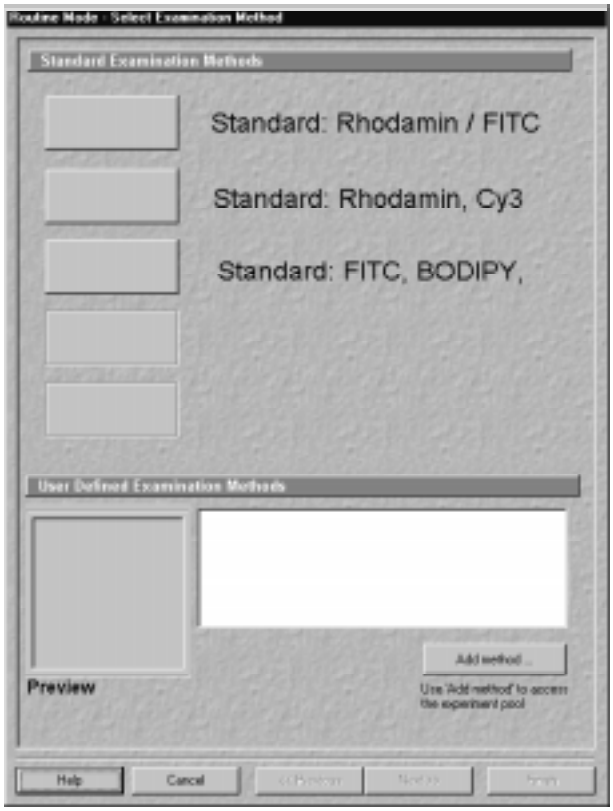


Fig. 4-74

4.6.2 Apply Standard Examination Methods in the Routine Mode

- Click on the button of the required standard examination method, e.g. Standard: Rhodamine / FITC.
  - The "Routine Mode - Microscope Setup" window will appear.
- Prepare your specimen for examination in the same way as in the Expert Mode.
- Click on the <Next> button.
  - The "Routine Mode - Image Setup" window will appear.

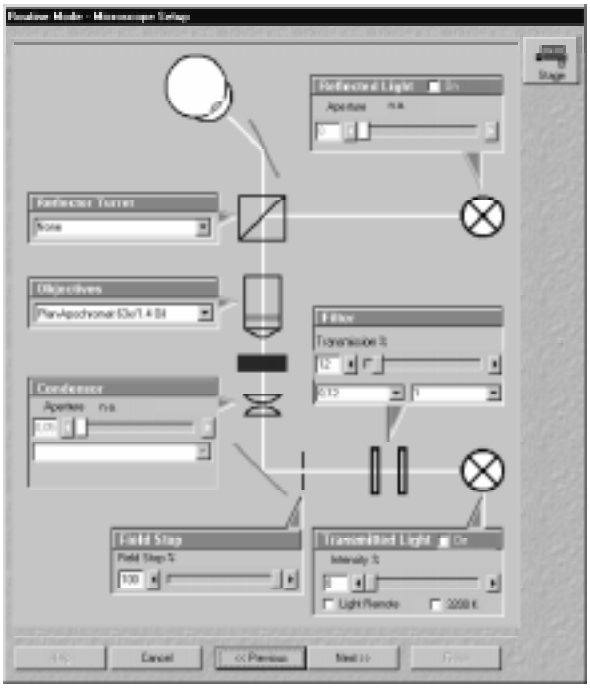


Fig. 4-75

- Click on <Single Scan> or <Cont. Scan>, to trigger the scanning procedure. If required, optimize the default parameters in the **Excitation**, **Zoom & Orientation**, **Image Quality Parameters** and **Noise Reduction** panels.
- Click on <Stop Scan> and <Next>.
  - The set parameters are displayed in the **Information For Method: Standard / FITC** panel.

☞ A bidirectional scan is not possible in the routine mode.

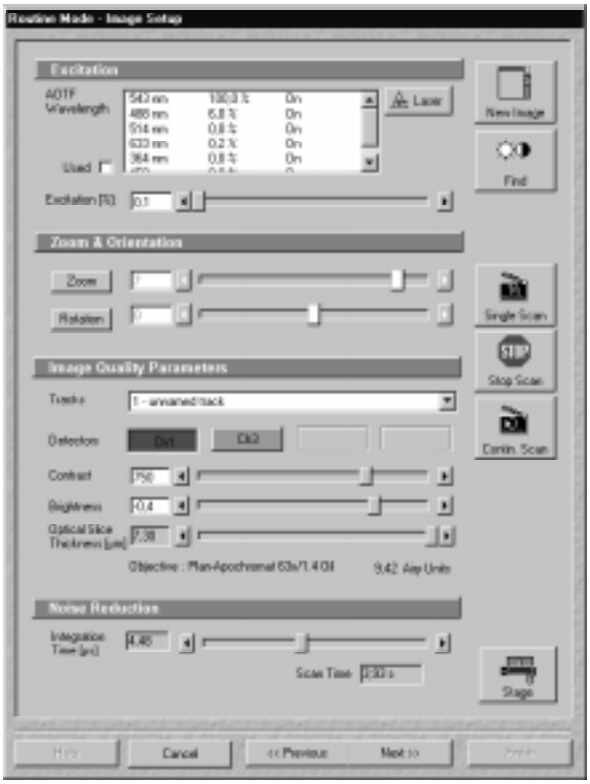


Fig. 4-76



Fig. 4-77

- Click on <Save> or <Save As> to store the acquired image.
- Click on <Finish> to exit the Routine Mode and return to the LSM 510 switchboard menu.

4.6.3 Export User-Defined Examination Methods to the Routine Mode

- Click on the <Start Expert Mode> button (Fig. 4-71) in the LSM 510 switchboard menu.
  - The "LSM 510 - Expert Mode" main menu appears on the screen.

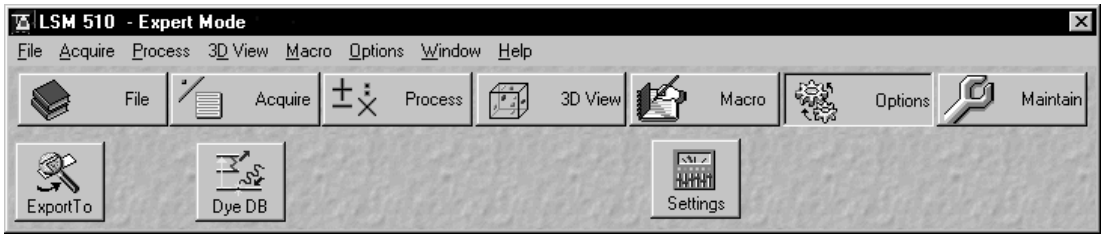


Fig. 4-78

- Load a Stack or Time Series image which was scanned using optimized parameters.

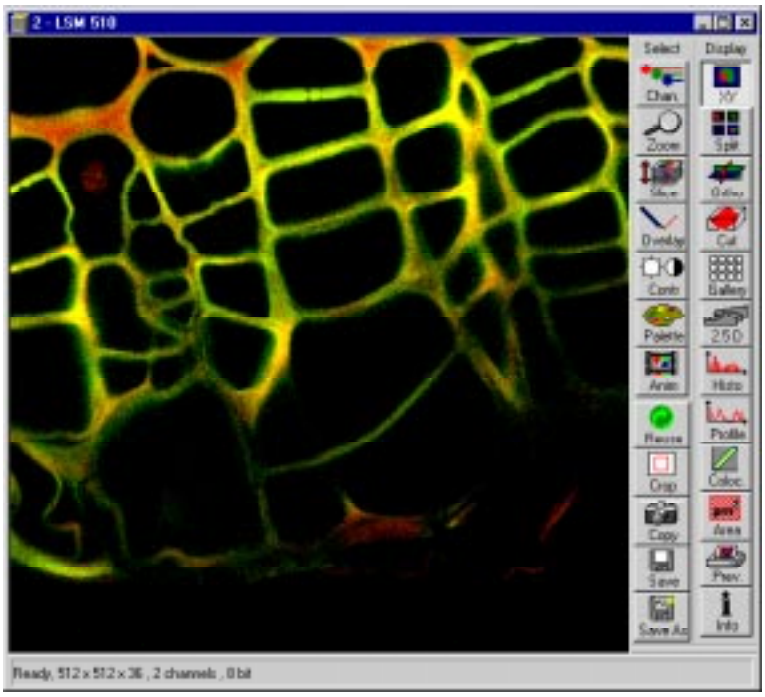
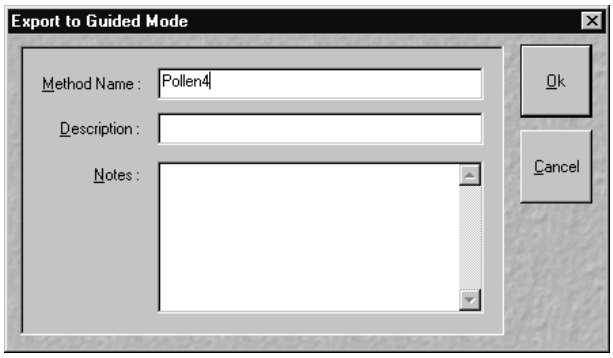


Fig. 4-79

- Click on the <Reuse> button (Fig. 4-79) in the opened image to take over the parameters.

- Click on the <Options> button of the main menu and then on <Export To> (Fig. 4-78).
  - The "Export To Routine Mode" window appears.



- Enter any name for the method to be taken over.
- Click on <OK>.
  - The method used for image acquisition is taken over in the Routine Mode.

Fig. 4-80

- Exit the Expert Mode by clicking on <File> and then on <Exit>.

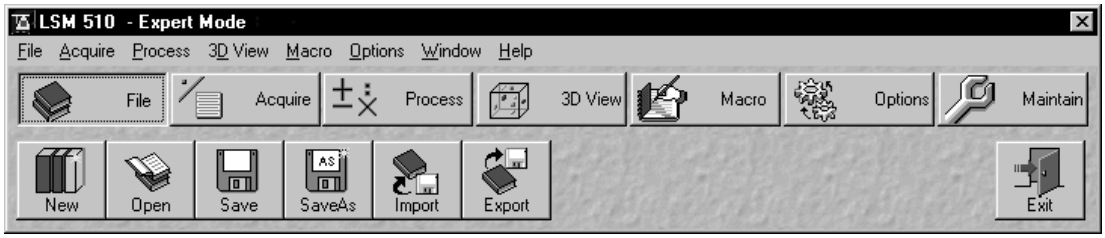


Fig. 4-81

  - The LSM 510 switchboard menu appears on the screen again.

4.6.4 Activate User-Defined Examination Methods in the Routine Mode

- Start the Routine Mode.
- Click on the <Add method> button.
  - The "Select Method To Add" window appears on the screen.

The method taken over from the Expert Mode appears, with the entered name, in the upper display field of the "Select Method To Add" window, though with an icon. The method can now be activated as **Standard** or **User-Defined Examination Method**.

- Click on the <Standard> or the <Mark for List> button.
- Close the "Select Method To Add" window by clicking on <Close>.
  - The "Routine Mode - Select Examination Method" window appears on the screen again. Depending on the allocation performed before, the method is assigned to a button of the Standard methods or appears in the list of **User-Defined Examination Methods**. The maximum of 5 methods can be defined as a standard and assigned to a button.

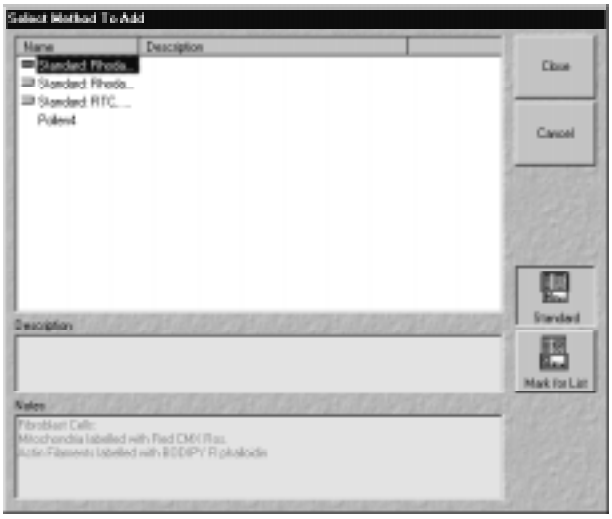


Fig. 4-82

4.6.5 Apply User-defined Examination Methods in the Routine Mode

- After a single click on the name entered in the list the method is displayed in the **Preview** box and can be started via <Next>.
- A double click on the name entered in the list activates the **Preview** display box and the method is started immediately.

User-defined Examination Methods are operated in the same way as the Standard methods.

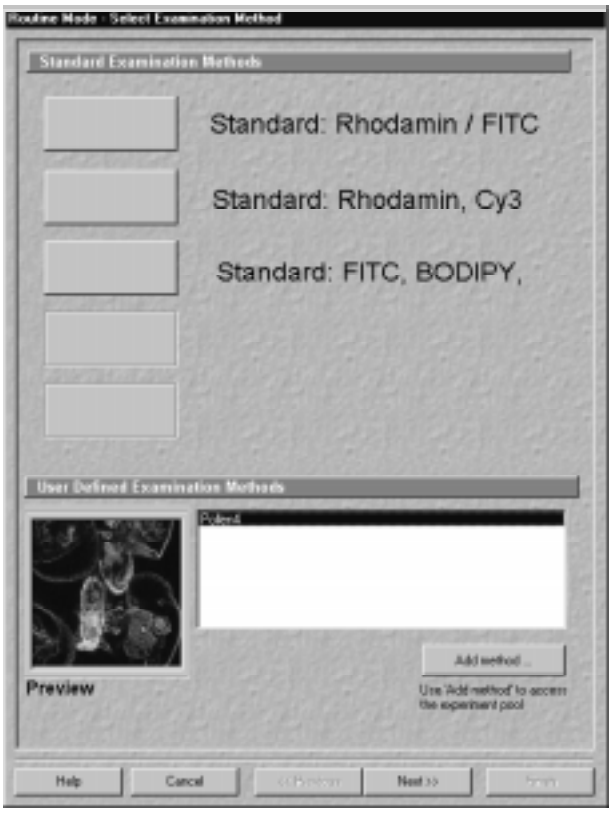


Fig. 4-83

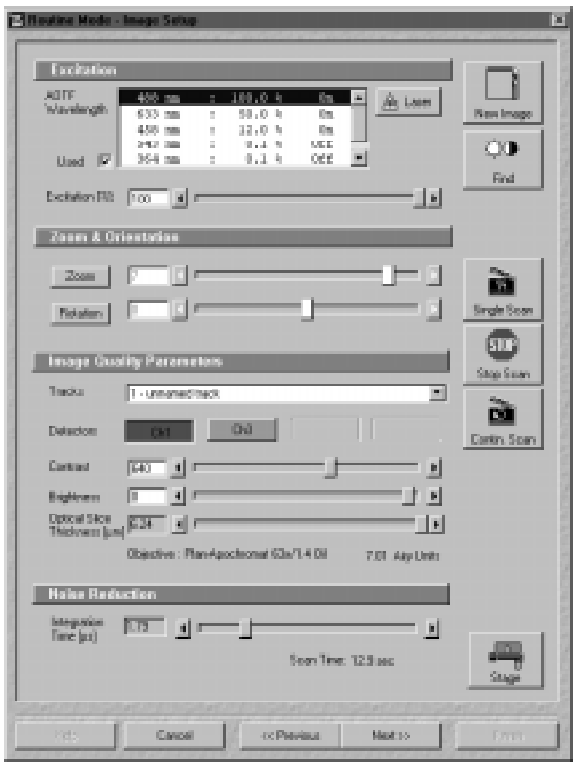


Fig. 84

4.6.6 Acquisition of a Z Stack in the Routine Mode

- Load the required stack, the parameters of which you want to use for further work.
  - The "Routine Mode - Microscope Setup" window becomes visible.
- Prepare your specimen for examination in the same way as in the Expert Mode.
- Click on the <Next> button.
  - The "Routine Mode - Image Setup" window becomes visible.
- You can optimize the scan parameters in the "Routine Mode - Image Setup" window.
- Click on the <Next> button.
  - The "Routine Mode - Image Stack Setup" window becomes visible.

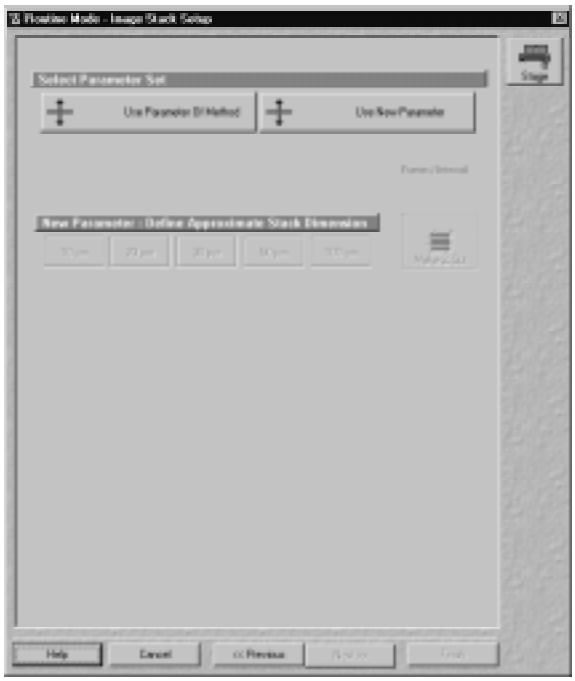


Fig. 85

- You can take over the offered parameters by clicking on the <Use Parameter of Method> button.
  - An x-y-frame of the stack center is produced and displayed.

- If you are satisfied with the setting, start by clicking on <Next>.
  - The "Routine Mode - Image Acquisition" window appears (Fig. 86).
- Click on <Start> to trigger acquisition of the stack.

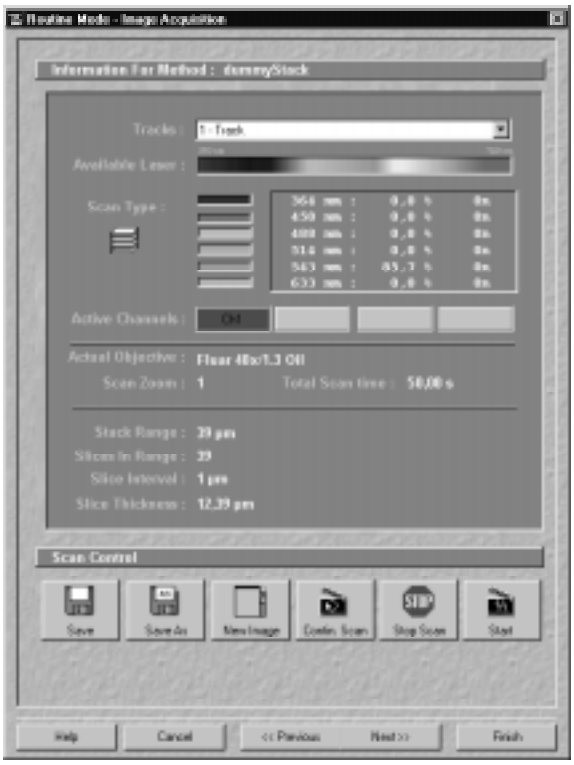


Fig. 86

- If you want to correct the parameters offered, click on the <Make Z Cut> in the "Routine Mode - Image Stack Setup" window button.
  - **Define Stack Parameter** panel appears in the "Routine Mode - Image Stack Setup" window

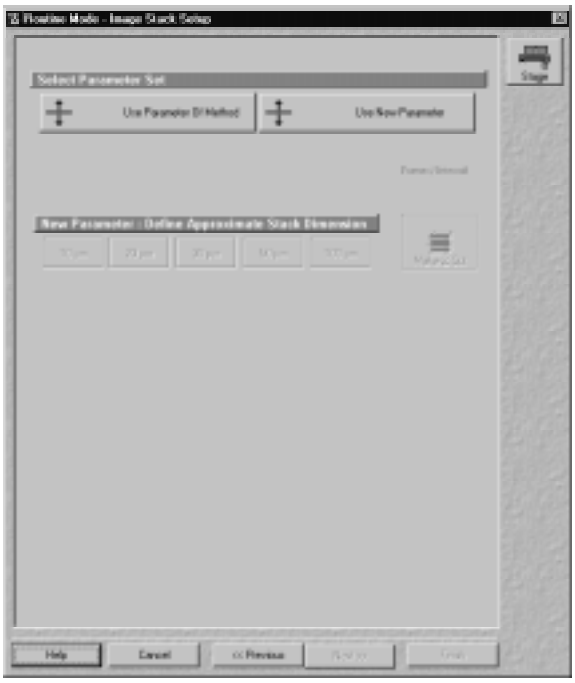


Fig. 87

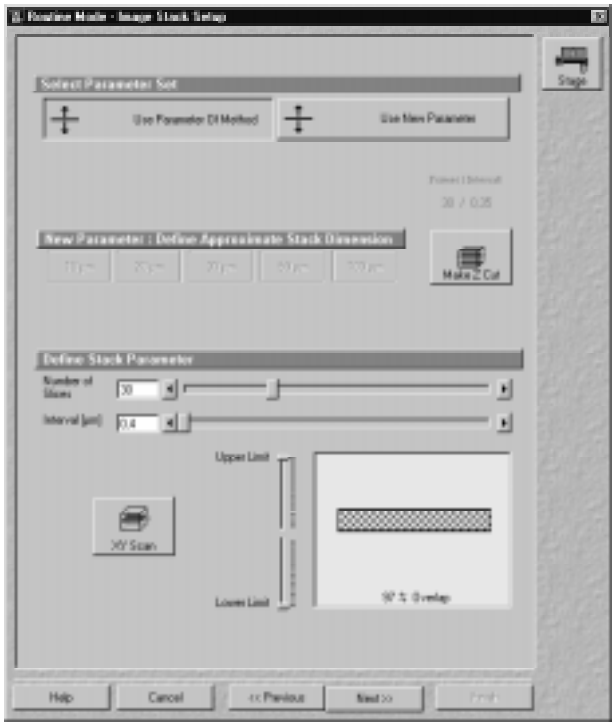


Fig. 88

- You can now select Z-stack limits (symmetrically to the central Z-frame), the number of steps and the interval size.

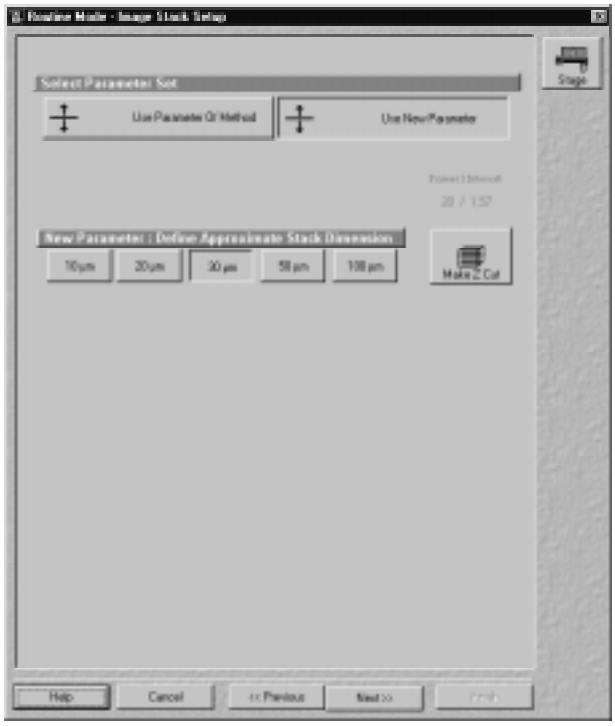


Fig. 89

Further modifications of the stack parameters can be performed as follows:

- Click on the <Use New Parameter> button.

It is possible to select from fixed stack sizes of 10 µm, 20 µm, 30 µm, 50 µm and 100 µm.

- After selection of the stack size, choose <Make Z Cut> or <Next>.
- Start acquisition of the stack by clicking on <Start> in the "Routine Mode - Image Acquisition" window.

4.7 Image optimization

Single frames

Described below is the example of the acquisition of an image, using an excitation wavelength of 488 nm and a fluorescence emission range above 505 nm.

Let the specimen be a thin section through a stem of Convallaria majalis (Lily-of-the-Valley). The description applies to the use of the Axioplan microscope, and analogously also to the Axiovert.

Requirements

- The suitable laser is switched on.
- The specimen has been positioned and focused for scanning.
- The slide rod on the microscope tube has been pulled out as far as it will go.

- Click on the <Config> button in the **Acquire** main menu item.
  - This opens the "Configuration Control" window.

Click on the <Recording Configuration> button. A window with the same title will open where you can select the relevant configuration and activate it via the <Apply> key.

- Open the list box.
- Select the necessary configuration (e.g. 488 nm, LP 505).
- Activate the selected configuration by clicking on the <Apply> button.

The **Beam Path and Channel Assignment** panel displays the configuration loaded.

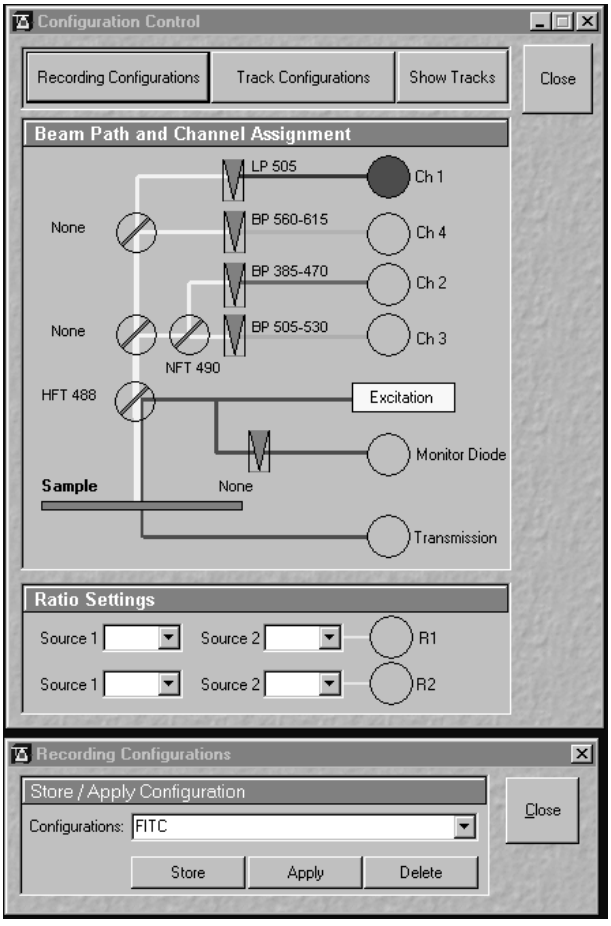


Fig. 4-90

☞ The set laser intensity can be subsequently optimized for the current situation via the <Excitation> button.

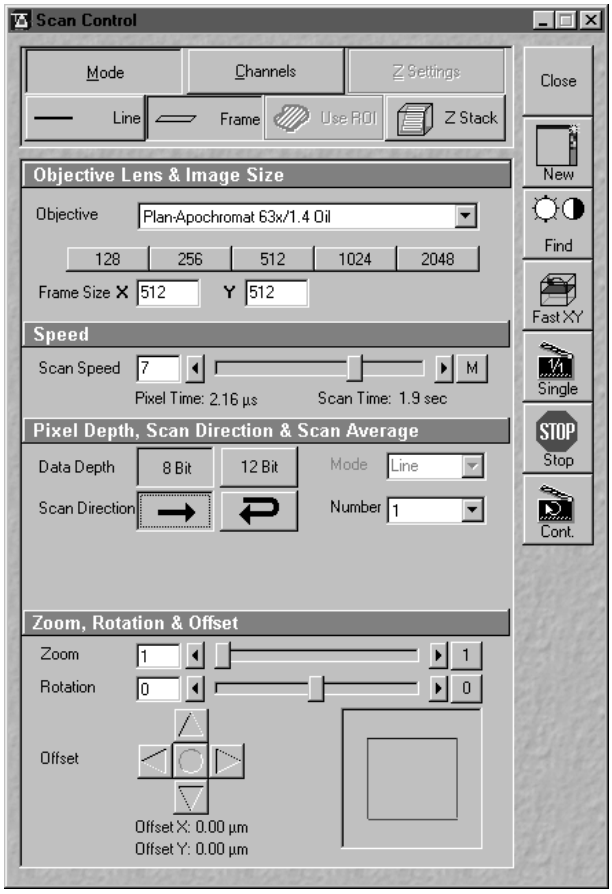


Fig. 4-91

- In the main menu click on the <Scan> button.
  – This opens the "Scan Control" window.
- Click on the <Mode > button.
- For a frame scan, click on the <Frame> button.
- On the **Objective Lens & Image Size** panel, select Objective and Frame size for the scan (e.g. X 512 / Y 512 scan)
- On the **Speed** panel, enter a scanning speed of e.g.7 to start with.
- Start with the following settings on the **Pixel Depth, Scan Direction & Scan Average** panel:
  Data depth: 8 bits
  Scan direction: → unidirectional
  Average: Number: 1
- On the **Zoom, Rotation & Offset** panel, set a Zoom of 1 and a Rotation of 0.

☞ Using the <Fast XY> button is a convenient way of creating an overview scan.

- Click on the <Channels> button.
  - This displays the preset parameters of the configuration loaded.

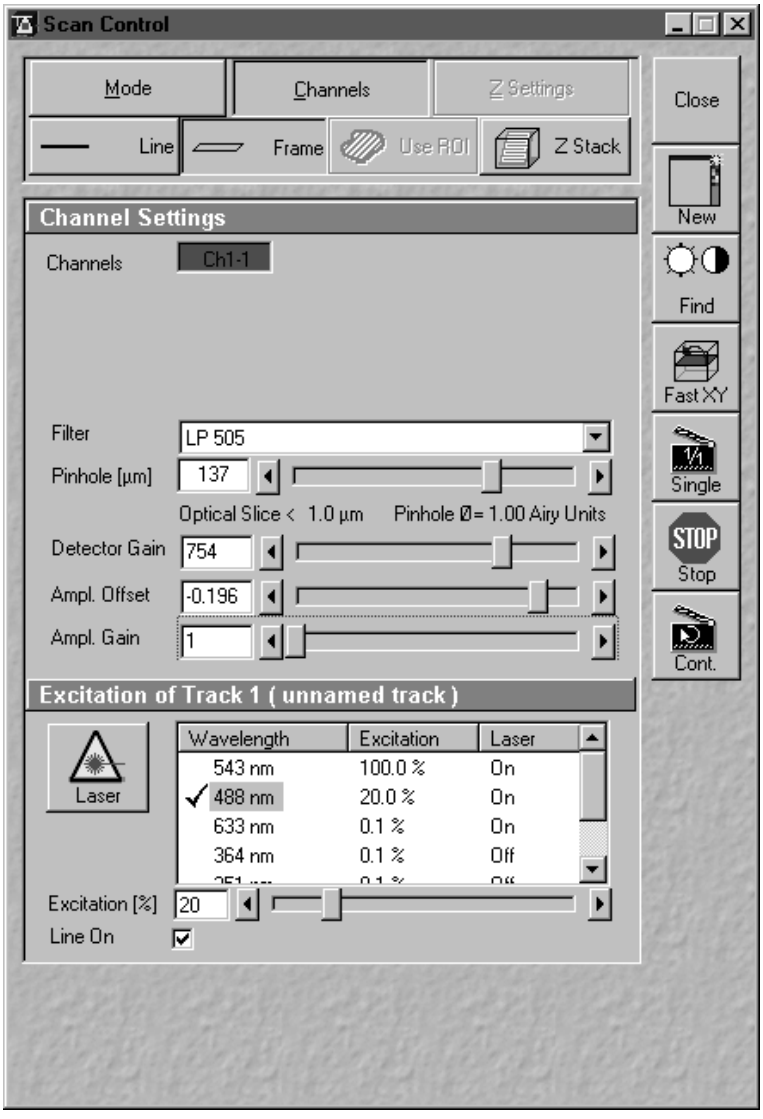


Fig. 4-92

- Click on the <Find> button. Make sure to position the slider correctly. Then scan while the slider is in the LSM position.
  - This starts the scanning process.
  - The image is seen to build up gradually in a new window.

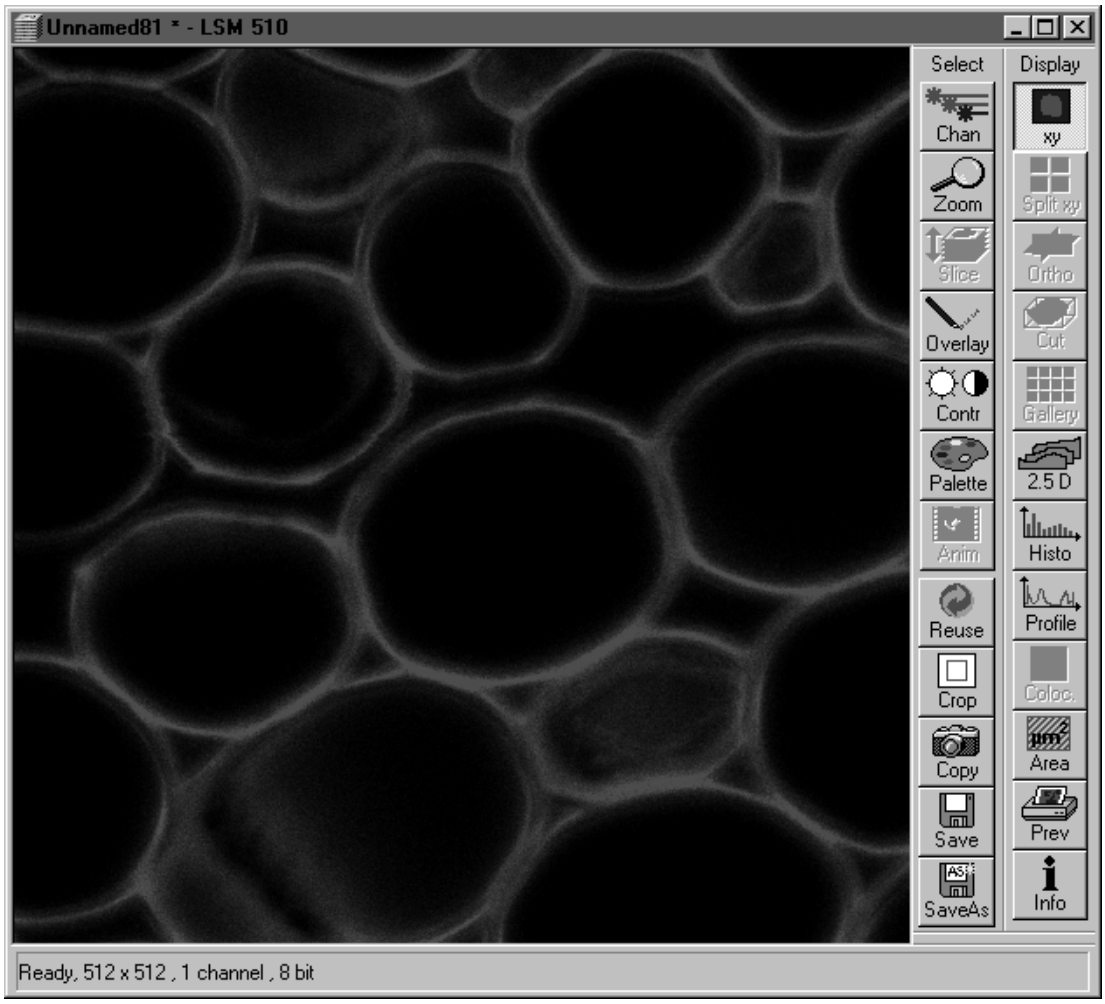


Fig. 4-93

As a rule, the first scanned image (Pre-Scan) is not ideal, since the photomultiplier is not matched to the light output. More often than not, the screen image is dull and needs subsequent optimization.

- Click on the <Single> button to release a single scan, or click on the <Cont.> button to release a continuous scan.

You can stop the scanning process with the <Stop> button at any time. Clicking on the <New> button opens a new window for the next scan.

4.7.1 Detector Gain/Ampl. Offset/Ampl. Gain

- In the "Scan Control" window, click on the <Cont.> button.
  - This starts a continuous scan.
- Click on the <Palette> button of the image processing toolbar.
  - This opens the "Color Palette" window.

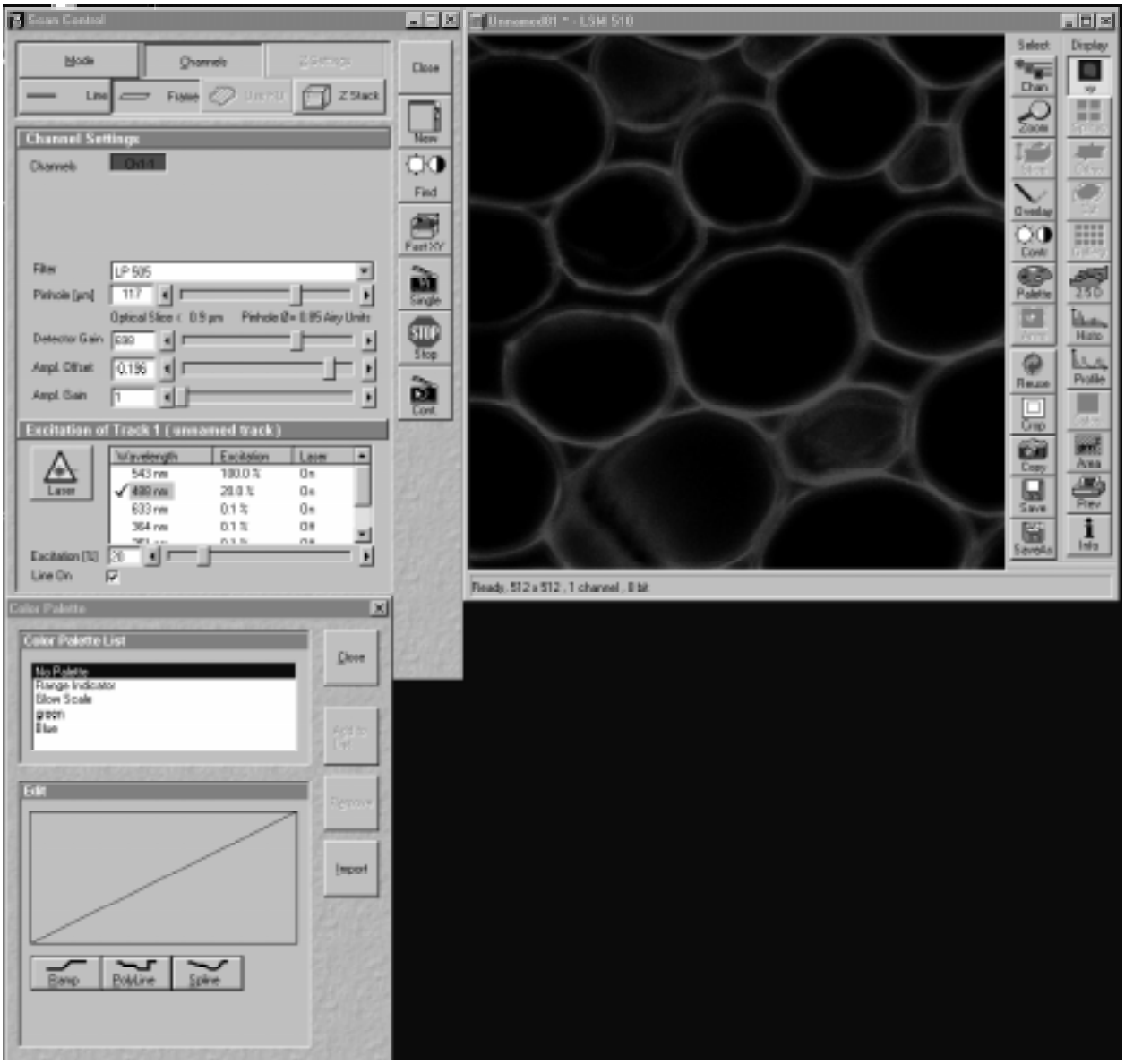


Fig. 4-94

- In the **Color Palette List** panel, click on the "Range Indicator" item.
  - The scanned image appears in a false-color presentation.

☞ If the image is too bright, it appears red on the screen.

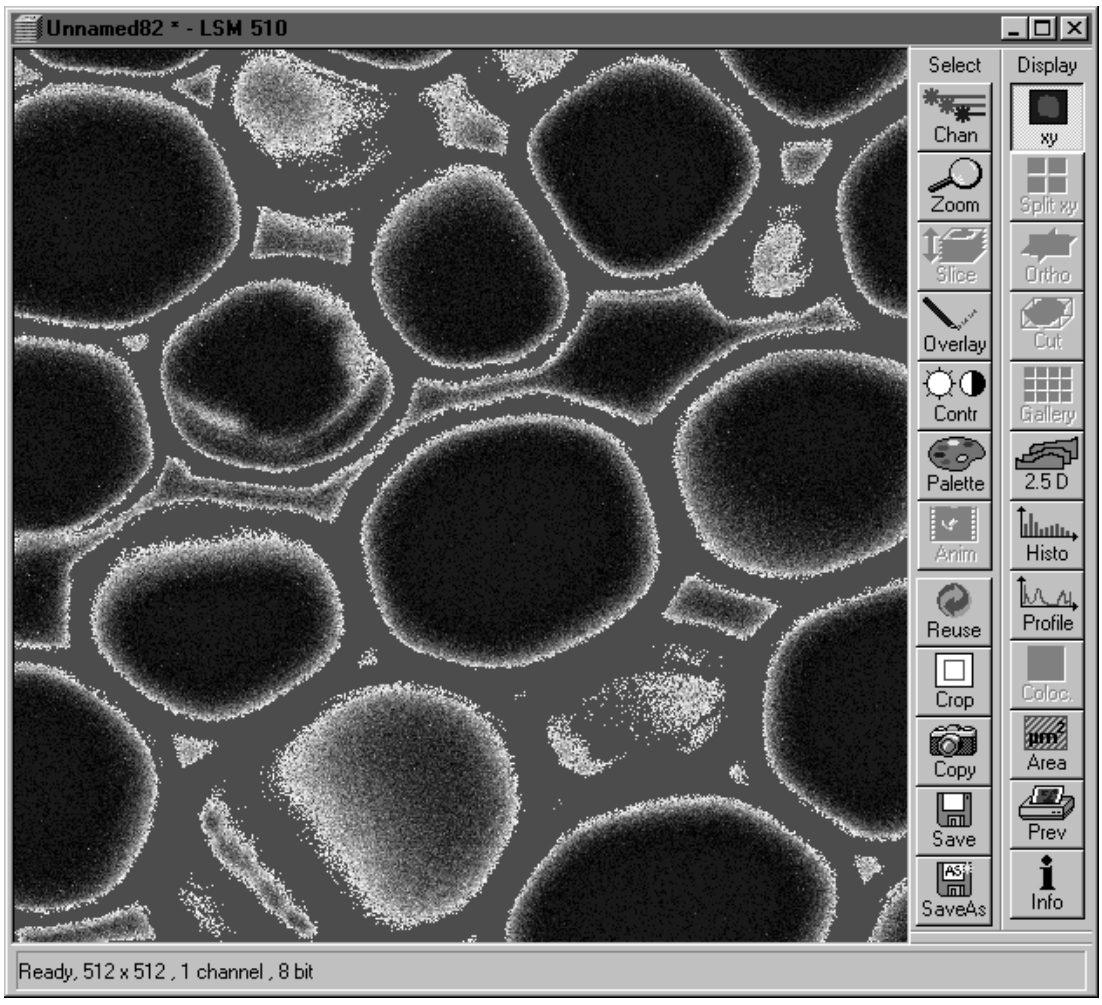


Fig. 4-95

☞ If the image is not bright enough, it appears blue on the screen.

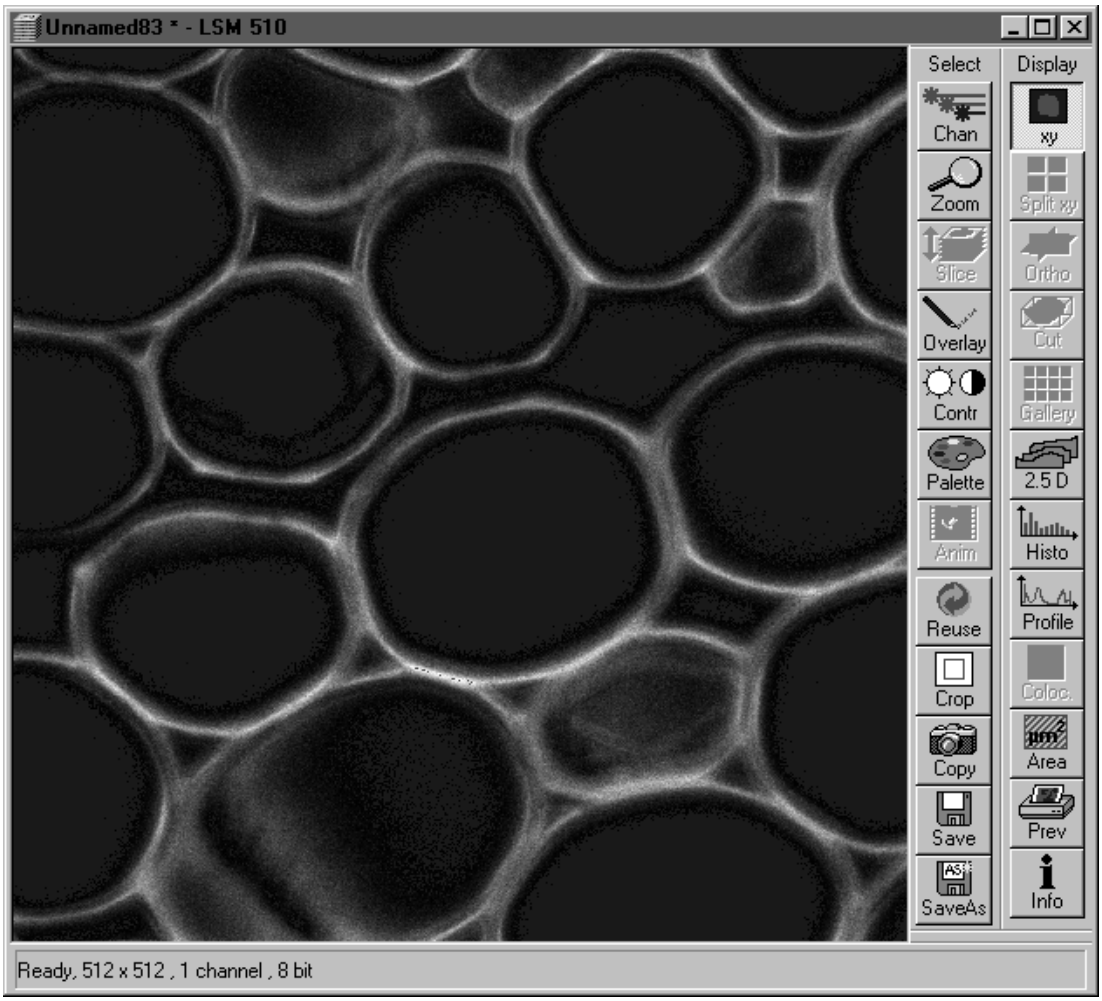


Fig. 4-96

- On the **Channel Settings** panel of the "Scan Control" window, set the PMT gain with the **Detector Gain** slider.
  - The image should not have more than a trace of red.
- Adjust the **Ampl. Offset** slider so that areas without picture content just show a trace of blue.
- If necessary, re-amplify brightness with the **Ampl. Gain** slider.

☞ Do not change the **Ampl. Gain** setting unless the settings made so far are insufficient for optimizing the image.

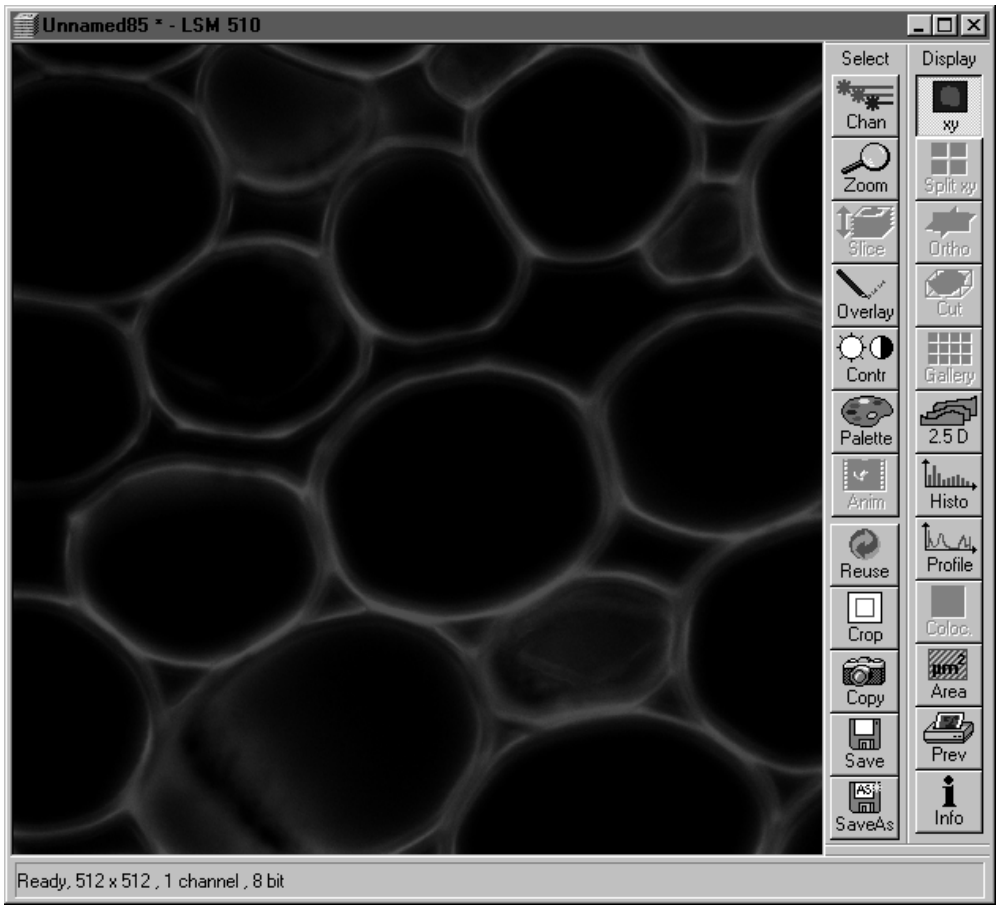


Fig. 4-97

- In the **Color Palette List** panel of the "Color Palette" window click on "No Palette".
  - This deselects the "Range Indicator" and activates the new presentation.
- In the "Scan Control" window click on the <Stop> button.
  - This stops the continuous scan.

4.7.2 Pinhole adjustment

☞ In all existing standard configurations, the pinholes have already been adjusted at the factory. These settings are taken over for active operation when a standard configuration is loaded.

If you want to create a setting that differs from the standard configurations, adjust the pinhole as follows:

Manual pinhole adjustment

The position of the pinhole relative to the detector in terms of X-Y-Z coordinates contributes substantially to image optimization.

Requirements to make pinhole position changes visible immediately:

- The image must be scanned by the continuous scan method.
- Select a fast scanning speed (see next section).
- Measurement with Average Number 1 only (no averaging of several measurements).
- On the **Channel Settings** panel (click on <Channels> button in the "Scan Control" window), select the pinhole diameter so as to have the best possible image contrast (Airy Units 1.0).

- Click on the <Maintain> button of the main menu toolbar.
  - This opens another, subordinate toolbar in the main menu.

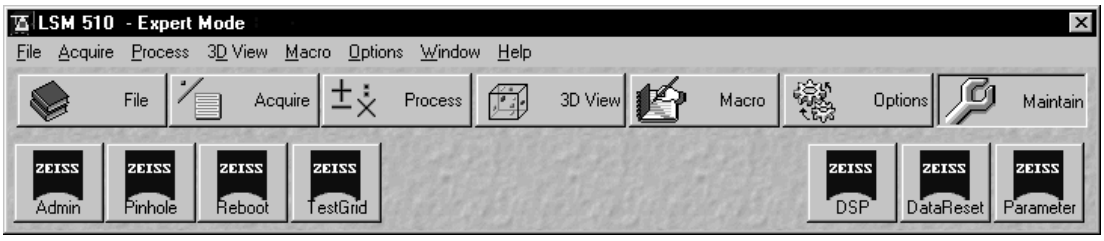


Fig. 4-98

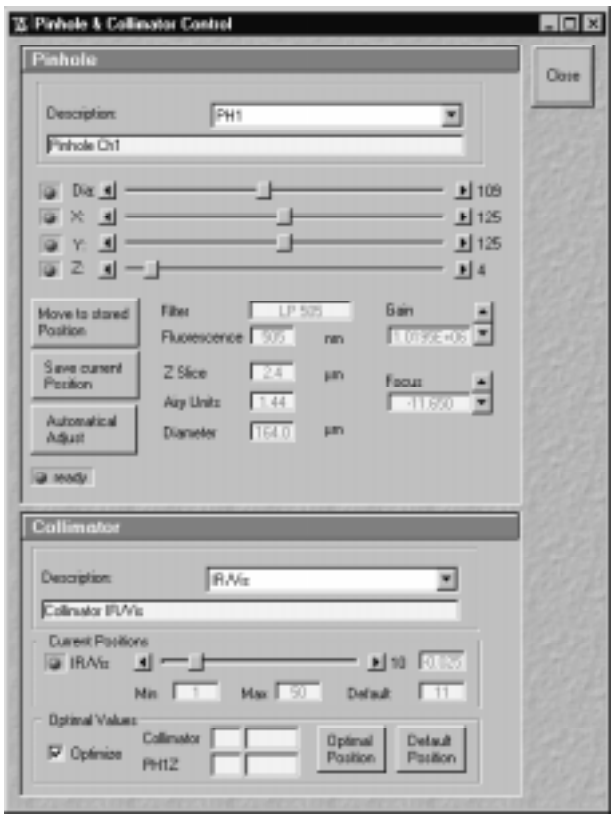


Fig. 4-99

- Click on the <Pinhole> button.
  - This opens the "Pinhole & Collimator Control" window.
- Select the pinhole to be adjusted from the **Description** list box.

☞ If several channels are used to produce the image, all the used pinholes must be adjusted separately.

- Use the **Dia**. (diameter) slider to set the smallest possible size which produces a good, high-contrast image.
  - This setting changes the pinhole diameter.
  - The **Z Slice** display box simultaneously displays the depth resolution corresponding to the pinhole diameter.

☞ Image optimization can be effected with the "Range Indicator" or in the **Line-Scan** mode.

- Optimize the pinhole position in X, Y and Z relative to the PMT with the X, Y and Z sliders to maximum image brightness.
- Click on the <Save current Position> button to save the pinhole adjustment.
- Removing the "Current Position" slider, allows the collimator to be adjusted to maximum image brightness. Optimum collimator adjustment received in this way can be stored by clicking on the <Save current Position> button.
- Click on the <Stop> button to stop the continuous scan.

Automatic pinhole adjustment and collimator

The position of the pinhole (X-Y-Z-coordinates) in relation to the detector makes a major contribution to image optimization.

In all of the available standard configurations, the pinhole positions are factory-adjusted and are activated when a standard configuration is loaded.

The automatic adjustment allows the LSM 510 pinholes to be used with any combination of beam splitters.

- Click on the < Adjust Automatically > button.
  - The **Requirements for Adjustment** dialog box will then appear.
- Meet the requirements listed in the dialog box and press the <OK> button
  - Pinhole adjustment will then run automatically. The adjusting procedure takes approx. 3 min.
  - The determined data are stored automatically and will be available for all further examinations using the same configuration

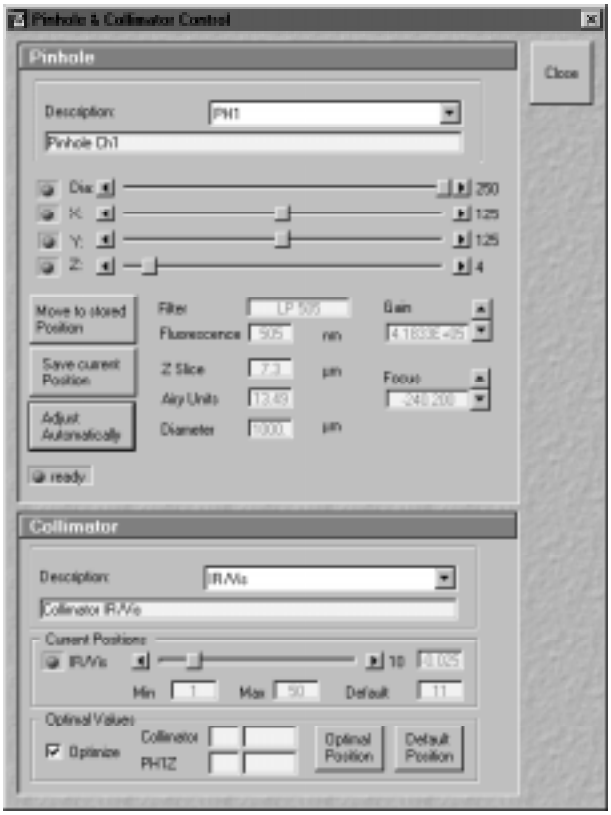


Fig. 4-100

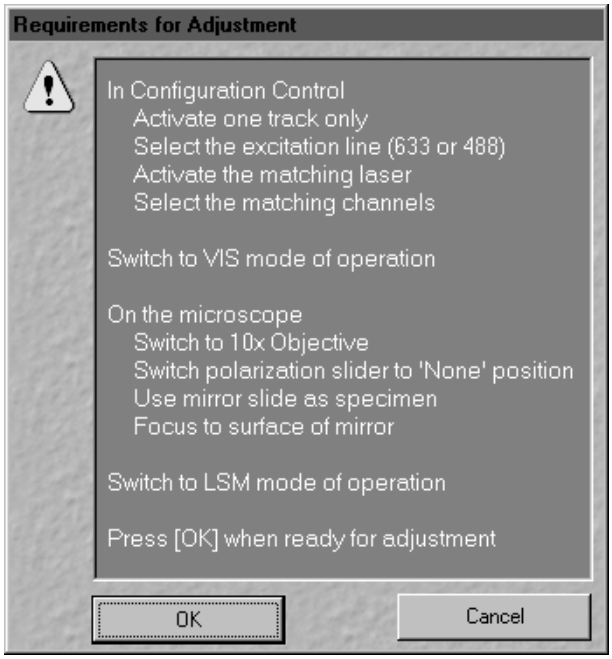

Requirements for Adjustment
In Configuration Control
Activate one track only
Select the excitation line (633 or 488)
Activate the matching laser
Select the matching channels
Switch to VIS mode of operation
On the microscope
Switch to 10x Objective
Switch polarization slider to 'None' position
Use mirror slide as specimen
Focus to surface of mirror
Switch to LSM mode of operation
Press [OK] when ready for adjustment
OK
Cancel

Fig. 4-101

- Click on the <Optimal Position> button in the **Collimator** box. Optimum positioning of the collimator will be performed. The <Default Position> button enables the collimator to be set back to the factory-adjustment.

Note:

A change of the pinhole diameter made manually in the **Pinhole** box will not be activated in the **Scan Control** box. Therefore, changes must always be made in the **Channel Settings** box.

A filter change in **Autoadjust** is not displayed in "Config. Control" window.

Configuration 1 is equipped in such a way that pinhole adjustment for channel 1 can only be made with λ = 488 nm, NFT 545, NFT 610 or NFT 570.

4.7.3 Scan speed

The signal-to-noise ratio can be substantially improved by reducing the scanning speed to an acceptable level and averaging over several scans (i.e. with an Average **Number** greater than 1 in the "Scan Control" window).

- Use the **Scan Speed** slider on the **Speed** panel to set the slowest acceptable scanning speed.
  - The corresponding pixel scanning time (Pixel Time) and the total scanning time (Scan Time) are shown in the dialog box.

- In the **Number** text box of the **Pixel Depth, Scan Direction & Scan Average** panel enter the number of measurements to be averaged.

☞ Image optimization can be effected much faster if you select a smaller frame, since less data have to be processed.

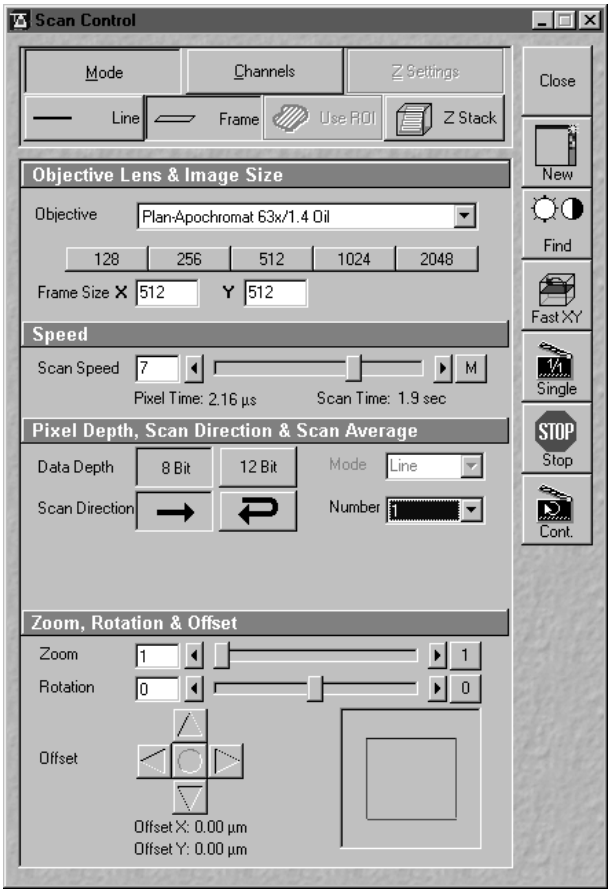


Fig. 4-102

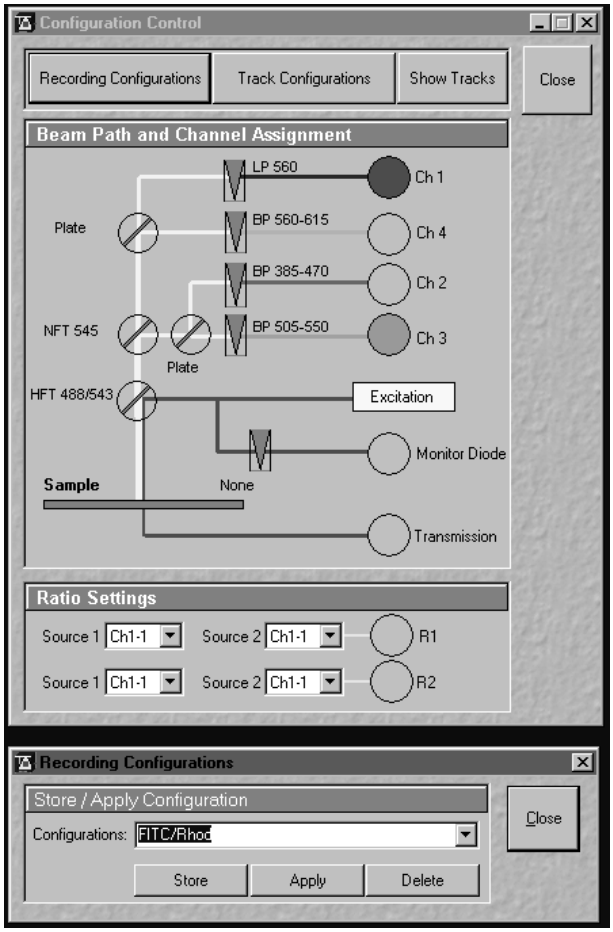


Fig. 4-103

Multiple-channel

Requirements:

– The suitable lasers are on.

– The specimen has been positioned and focused for scanning.

– The slide rod on the microscope tube has been pulled out as far as it will go.

- In the "Acquire" main menu item, click on the <Config> button.
  – This opens the "Configuration Control" window.
- Select the necessary configuration by clicking the <Recording Configurations> button, selecting a proper configuration and pressing the <Apply> button (e.g. 488 nm/543 nm).
  – The configuration loaded is displayed on the **Beam Path and Channel Assignment** panel.
- Close the "Recording Configurations" window.

☞ The laser intensity set can be subsequently optimized for the job via the <Excitation> button.

- Click on the <Scan> button in the main menu.
  - This opens the "Scan Control" window.
- In the "Scan Control" window, set the parameters in the same way as described for single-channel presentation.
- Click on the <Find> button in the "Scan Control" window.
  - This starts the scanning process. The scanned image appears in a separate window.

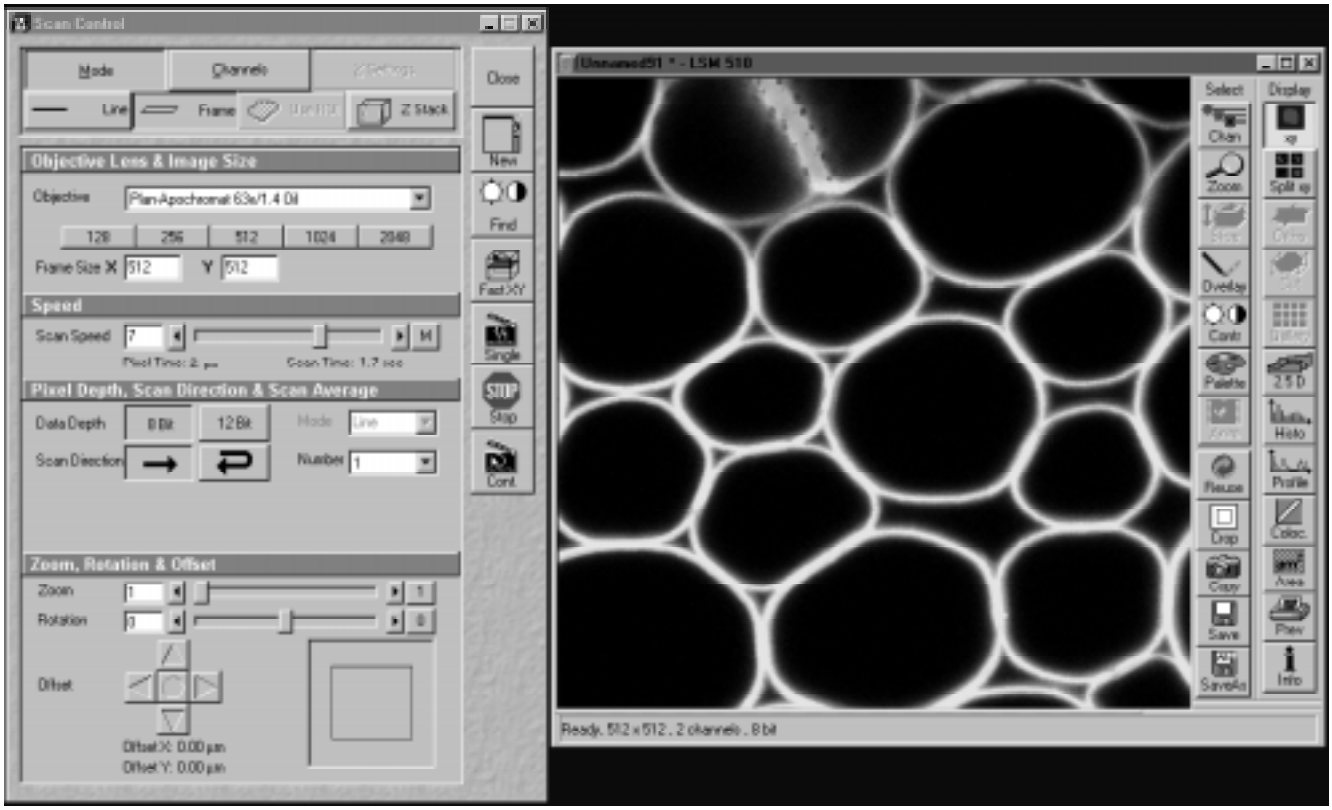


Fig. 4-104

☞ As a rule, the first scanned image (Pre-Scan) is not ideal, since the photomultiplier is not matched to the light output. More often than not, the screen image is dull and needs subsequent optimization.

- Click on the <Single> button to release a single scan, or on the <Cont.> button to release a continuous scan.

You can stop the scanning process with the <Stop> button at any time. Clicking on the <New> button opens a new window for the next scan.

- Click on the <Channels> button in the "Scan Control" window.
  - This opens the **Channel Settings** and **Excitation of Track** panels.
  - The channels used are color-highlighted.

The image optimization processes

- **Detector Gain** / **Ampl. Offset** / **Ampl. Gain**
- Pinhole adjustment
- Scanning speed and Average

must be carried out separately for each channel used.

- Click on the <Cont.> button in the "Scan Control" window.
  - This starts a continuous scan.
- Now click on the <Ch1> button on the **Channel Settings** panel.

- Click on the <Display - Split xy> button of the image processing toolbar.
  - This displays the separate images scanned in the channels and the composite image.

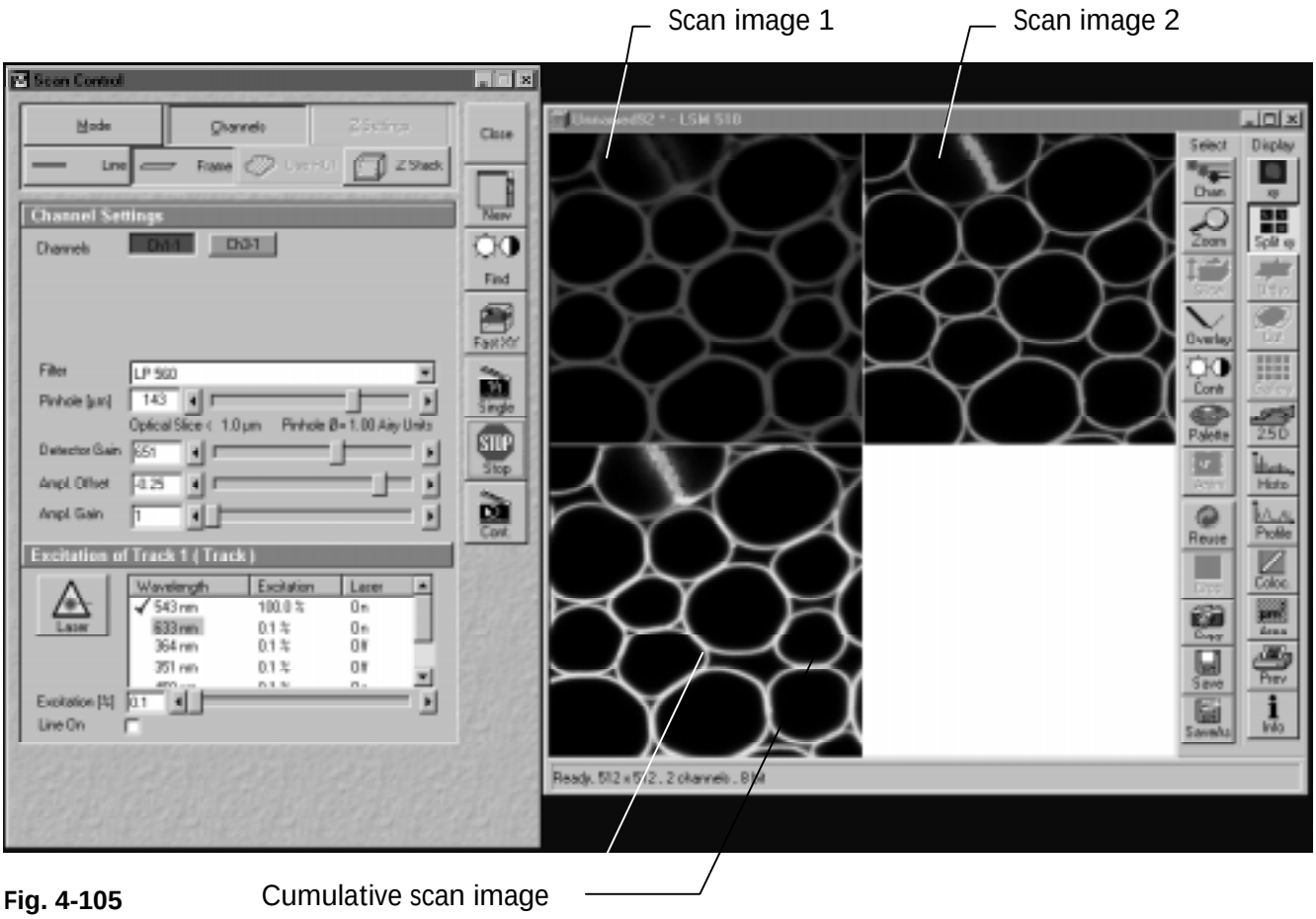


Fig. 4-105

Now effect image optimization as explained for the single-channel mode, but separately for each channel.

- Now click on the <Display - xy> button.
  - The composite scan image of two channels is presented in a common window.

☞ Image optimization can be effected much faster if you select a smaller frame, since less data have to be processed.

4.7.4 Channel Shift Function

The **Channel Shift** function is used to produce a congruent image with relation to the pixels of the various channels.

This pixel correction function is particularly important in UV applications.

(1) Click on the <Process> and <Shift> buttons from the LSM 510 tool bar. The **Channel Shift** menu appears on the screen.

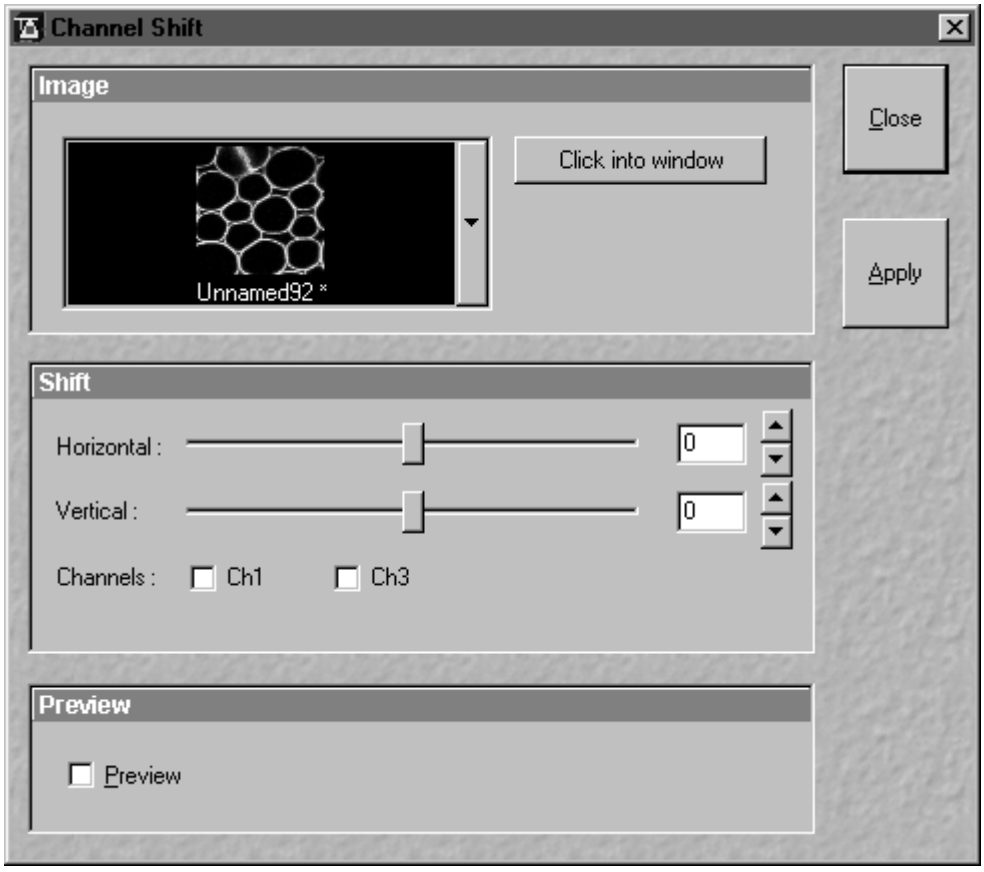


Fig. 4-106

(2) Select the channels required for processing in the **Shift** box by clicking on the <Ch1> or <Ch3> buttons. A tick ☑ will appear in the button when the channels are activated.

(3) Use the scrollbar or the < ▲ > and < ▼ > buttons to select the pixel shift in the horizontal and vertical direction.

(4) Click on the <Apply> button to activate the setting. If <☑ Preview> is activated, a preview of the shift is shown in a separate image window.

The following image shows the result of a pixel shift via the **Shift** function. This image change can be stored in the image database via the <Save> or <Save As> buttons.

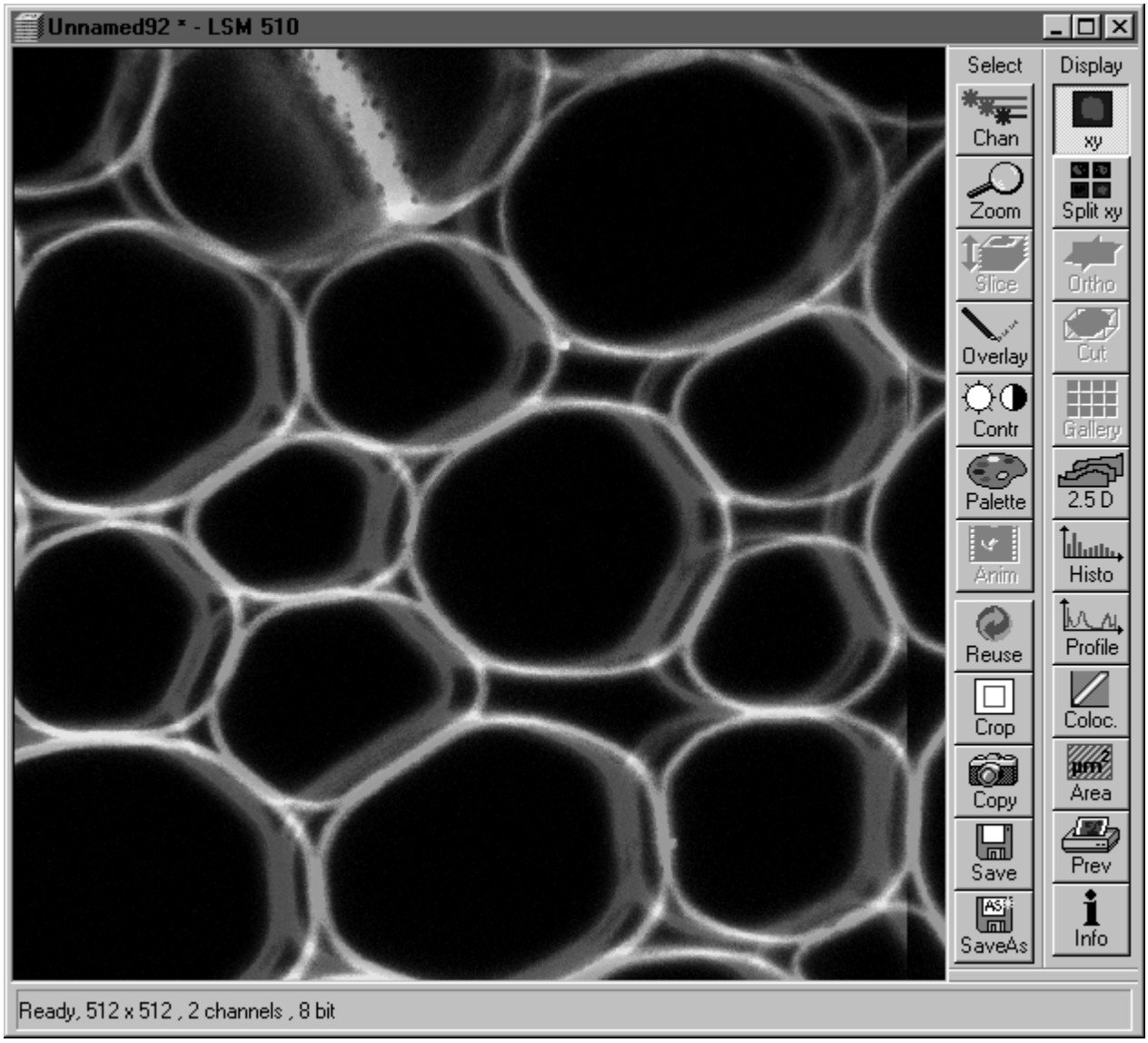


Fig. 4-107

For applications requiring 3- or 4-channel scanning, proceed in the same way as described for the 1- or 2-channel mode.

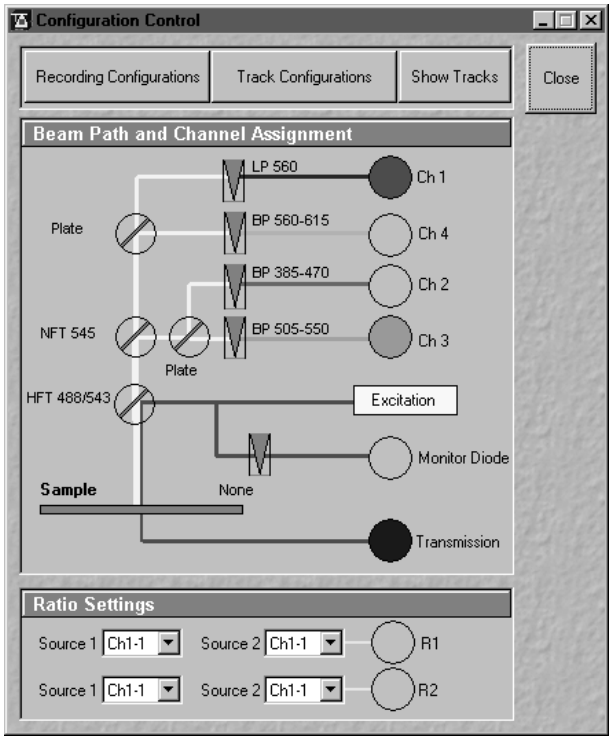


Fig. 4-108

For overlaying fluorescence and transmitted-light images, click on the <Transmission> button on the **Beam Path and Channel Assignment** panel.

The transmitted light PMT will be switched active.

Of course, all other transmitted light applications like

- phase contrast
- differential interference contrast (DIC)
- polarization contrast (Pol)
- darkfield

can also be performed.

Image optimization must be performed as described in the previous chapter.

For the generation of images in reflection, the main dichroic beam splitter must be a neutral-density filter.

Standard equipment contains a neutral-density filter with a division ratio of 80 to 20 % (at 543 nm).

4.8 Analysis of Images and Stacks

- Call up an image just scanned, or an image from the database.



Fig. 4-109

- The image information called up is shown in a window. The two columns of buttons headed "Select" and "Display" of the toolbar on the right allow you to select a wide variety of image post-processing actions.

(1) Select - Chan

You can assign any color from the color palette to a channel, so as to highlight or suppress certain image details.

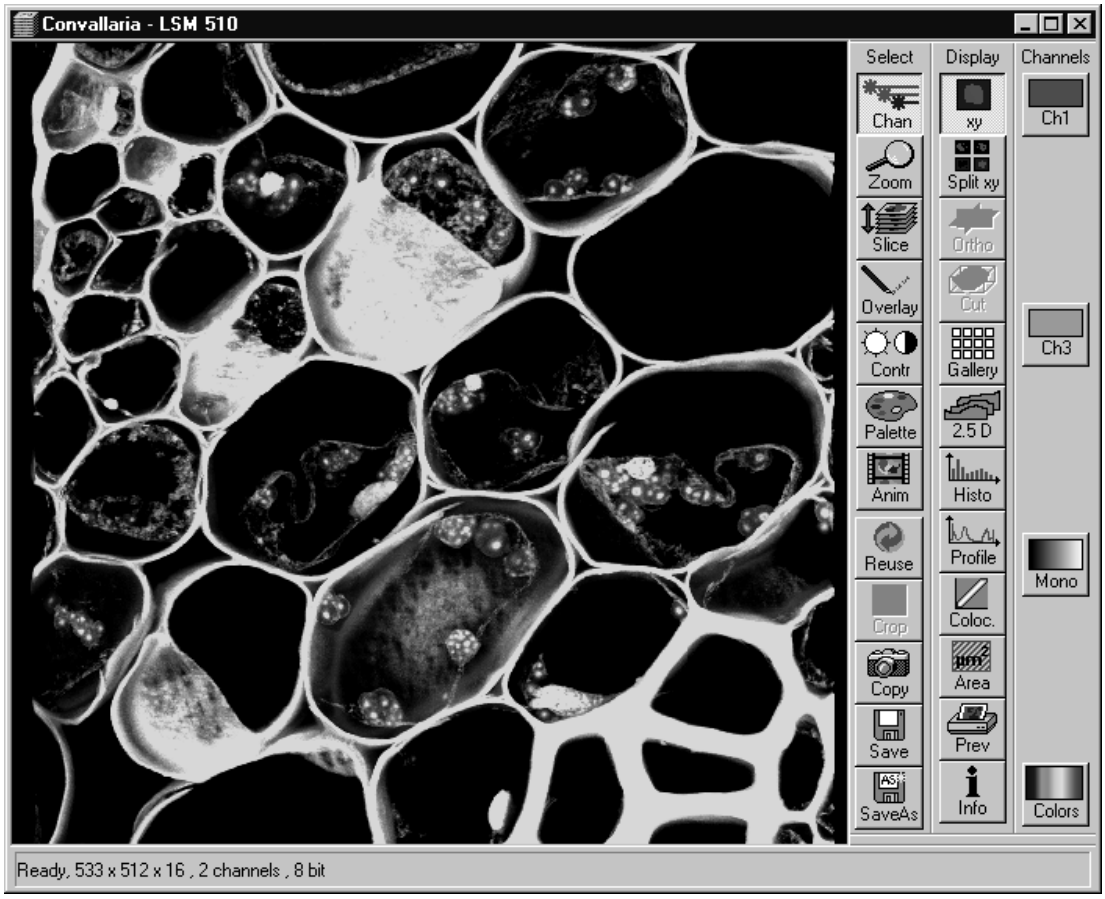


Fig. 4-110

(2) Select - Zoom

The image can be zoomed by various methods.

Zoom-Auto

The scanned image is automatically zoomed to the window size.

Resize

Restores the image to its initial size.

☞ **Zoom-+, Zoom-–, Zoom 1:1** and **Zoom-Mouse** can only be defined when the **Zoom-Auto** function is deactivated.

Zoom-+

Enlarges the image by factor 2.

Zoom-–

Reduces the image by factor 2.

Zoom 1:1

Restores an image zoomed in any way to its original size.

Zoom-Mouse

Allows you to enlarge/reduce an image using the left/right mouse button, provided that the cursor is inside the image.

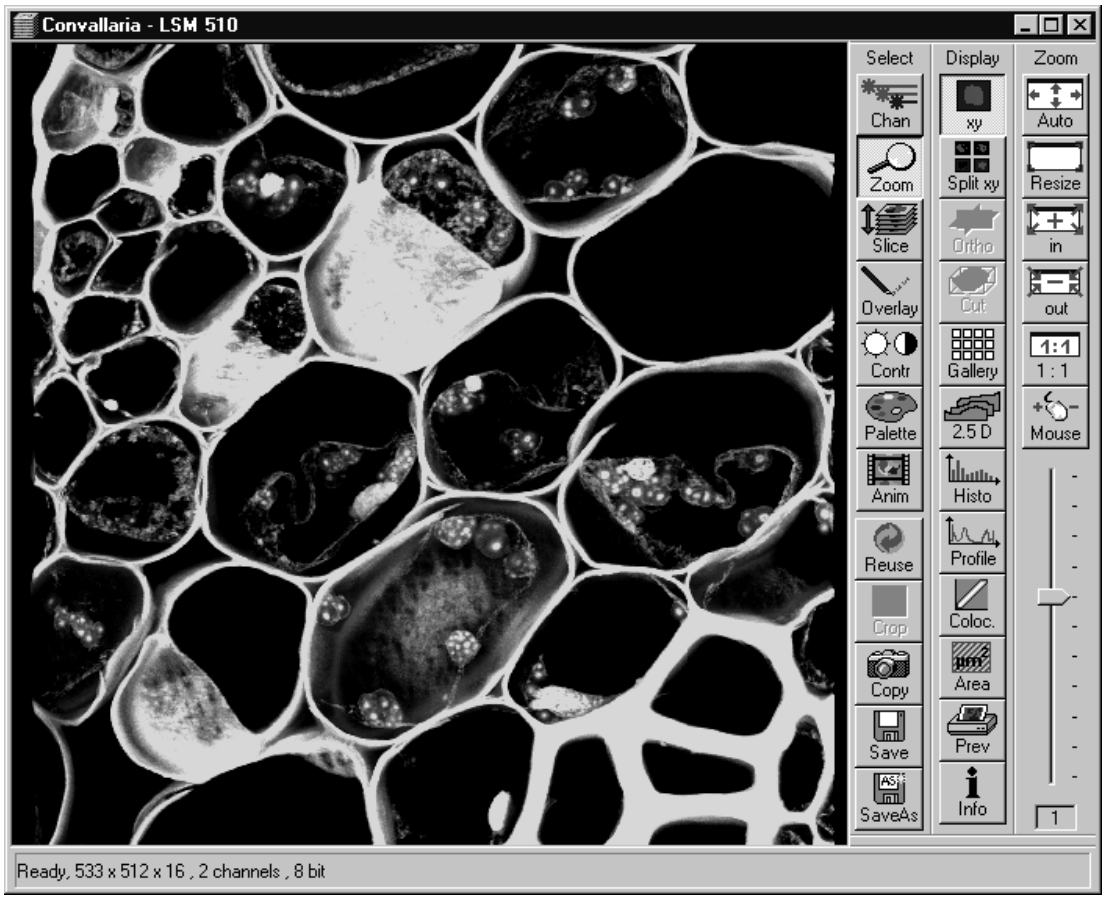


Fig. 4-111

(3) Select - Slice

This function allows you to select and view individual slices from a stack.

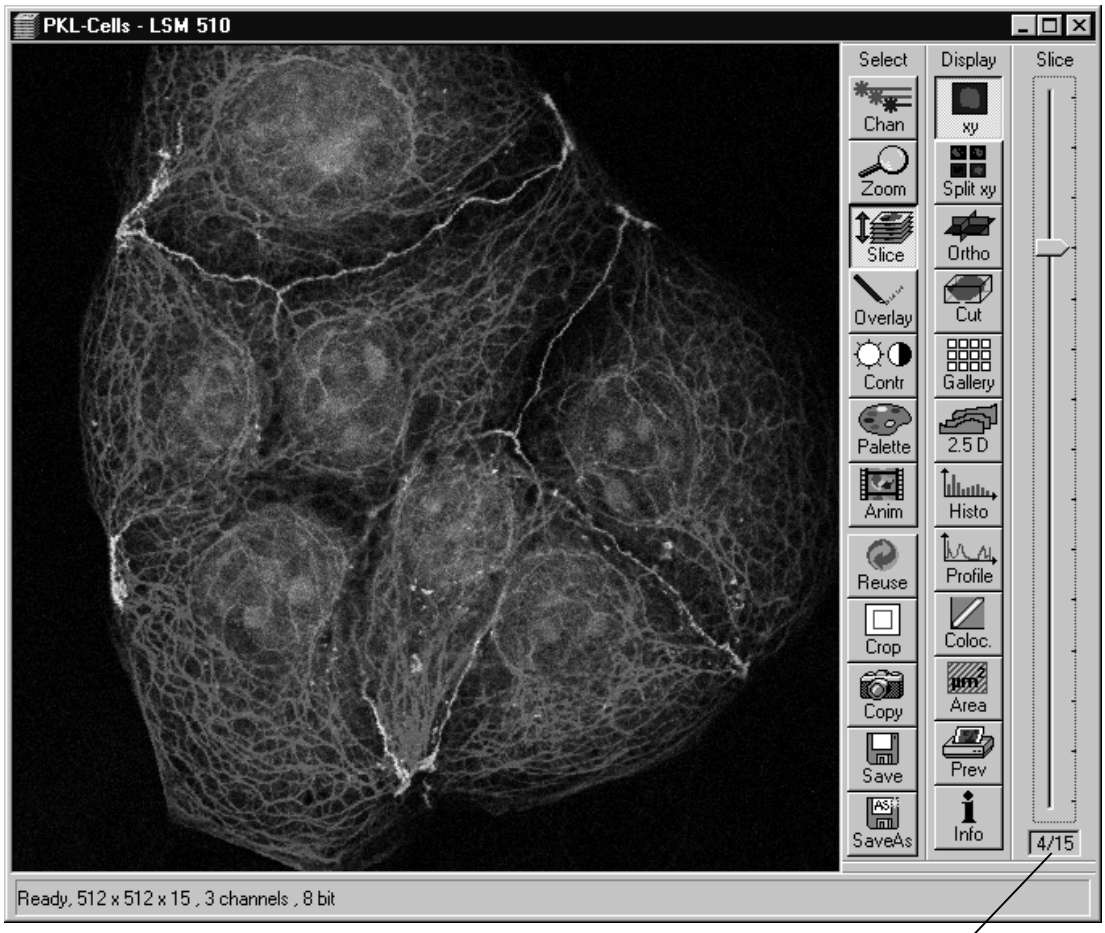


Fig. 4-112

Example:
Slice No. 4 from a stack of 15 slices

Select the slices using the slider on the right.

(4) Select - Overlay

This option makes available a number of measurement functions, such as length, angle, area and circumference measurements. Furthermore, you can enter comments into the image.

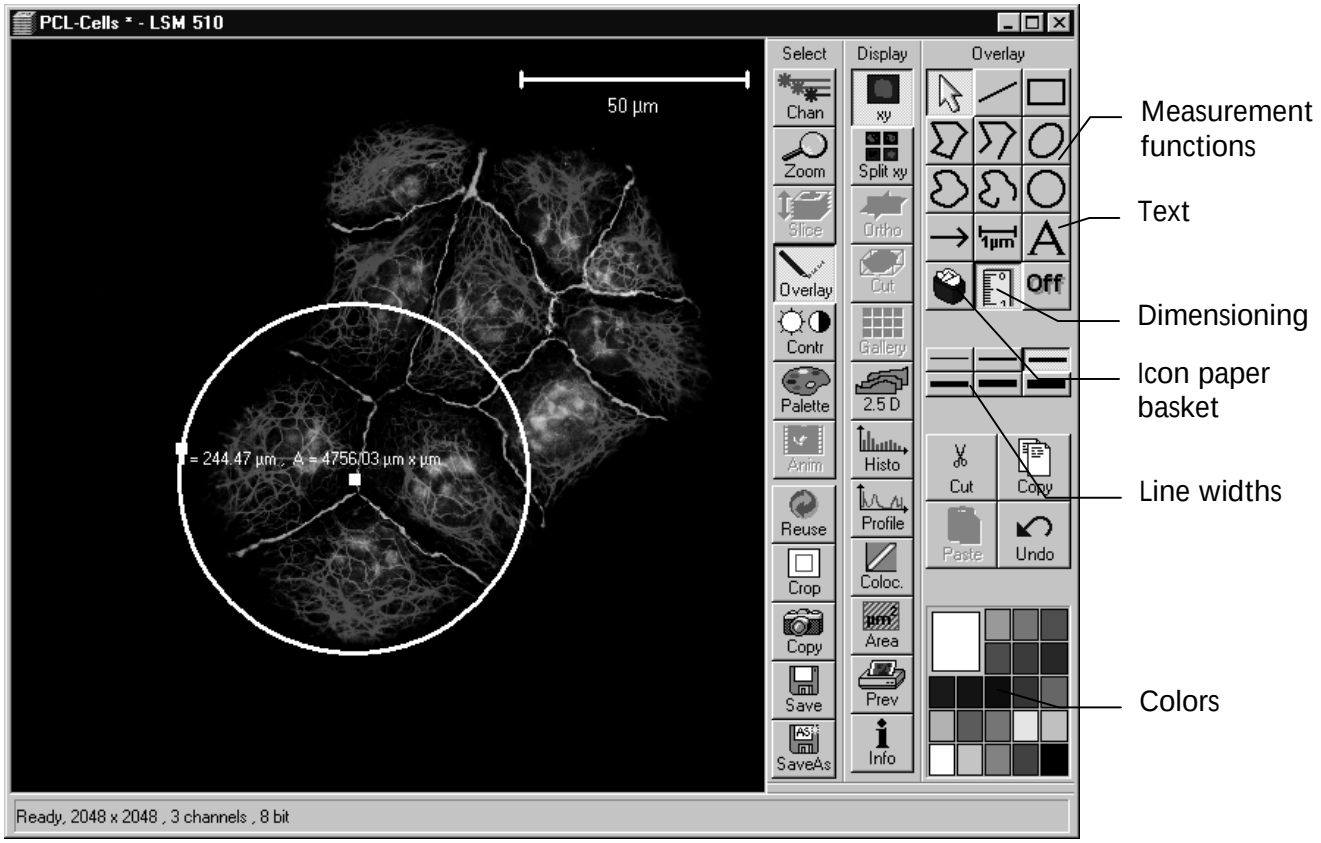


Fig. 4-113

Using the "paper basket" icon you can delete markings and dimensions in the image.

☞ After an objective change, a new scan must be performed to receive correct measurement results in the actual frame.

(5) Select - Contr

This function allows you to vary the contrast and brightness of an image, either separately for each channel or jointly for all channels. Further image processing possibilities can be activated or deactivated alternately using the <More> and <Less> buttons.

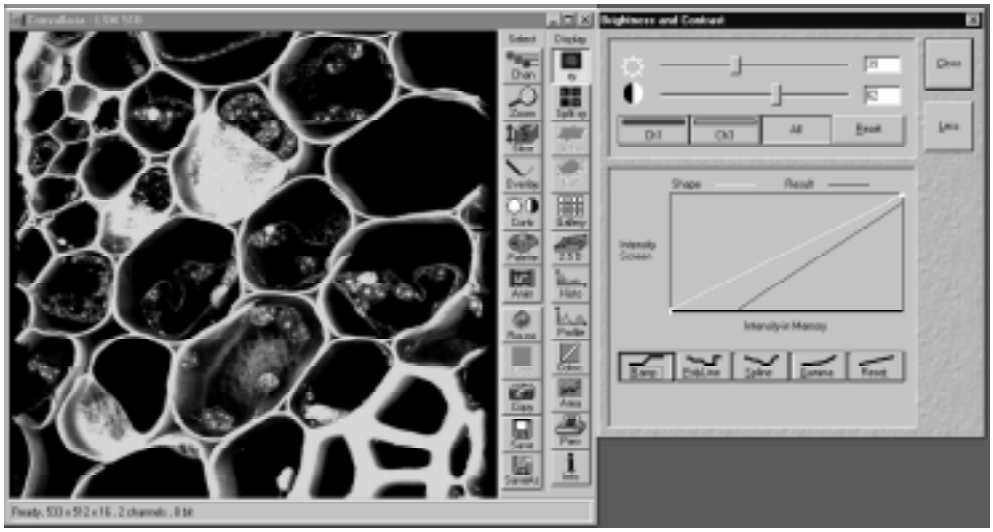


Fig. 4-114

(6) Select - Palette

This option allows you to view the image in different color presentations. The image can be modified according to mathematical functions.

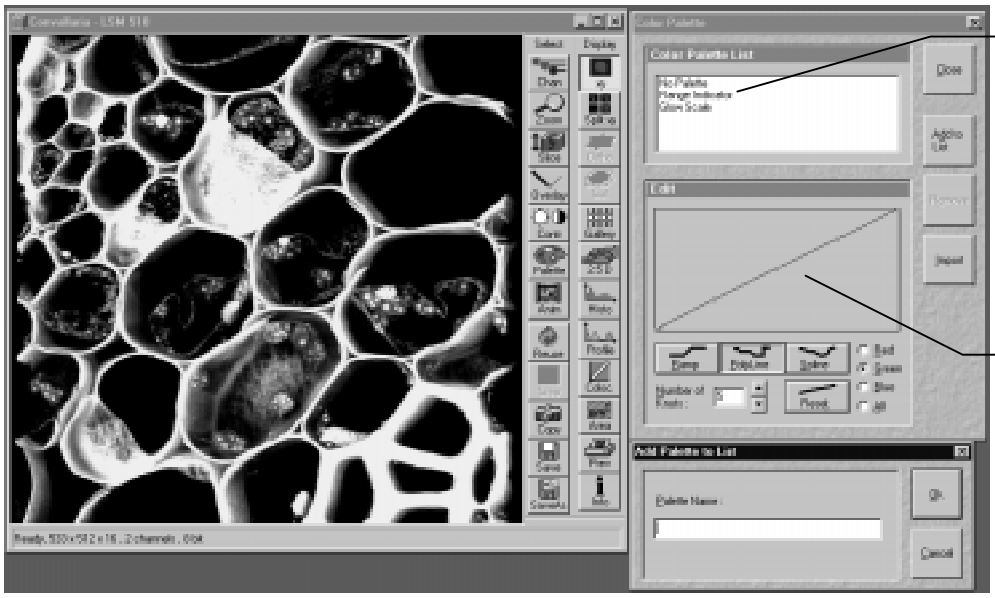


Fig. 4-115

(7) Select - Anim

This option allows you to call up the individual slice images of a stack in continuous succession (animation).

The additional **Animate** dialog box allows you to influence the animation action.

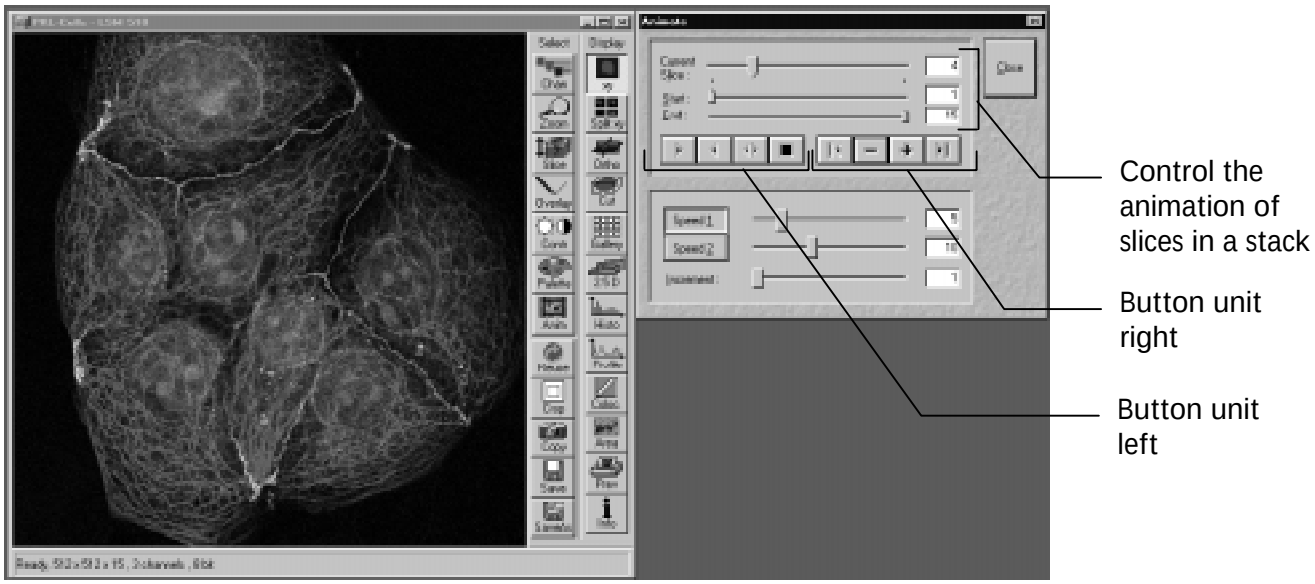


Fig. 4-116

Using the left button unit, the stack can be passed automatically. The right button unit allows you to leaf through the stack manually.
Using the <Speed1> and <Speed2> buttons, the preadjusted running speed can be interrogated.
With the <Increment> function, you can define the step width within the stack.
Only these step-by-step images will be shown in a running stack sequence.

(8) Select - Reuse

The parameters used for the creation of an image are stored together with the relevant image data. The <Reuse> function allows the parameters of the loaded image to be automatically set in the instrument and to continue working with this setting.

(9) Select - Crop

Here, you can define an area of any size, position and rotation which you want to scan.

Offset:

- Click into the square (scanning field), keep the left mouse button pressed and drag the square to the required position. Release the mouse button.

Zoom:

- Click on a corner of the square, keep the left mouse button pressed and set the required size. Release the mouse button.

Rotation:

- Click on one end of the crosslines, keep the left mouse button pressed and set the required rotation angle. Release the mouse button. For better orientation during the rotation, one side of the square is highlighted in blue.

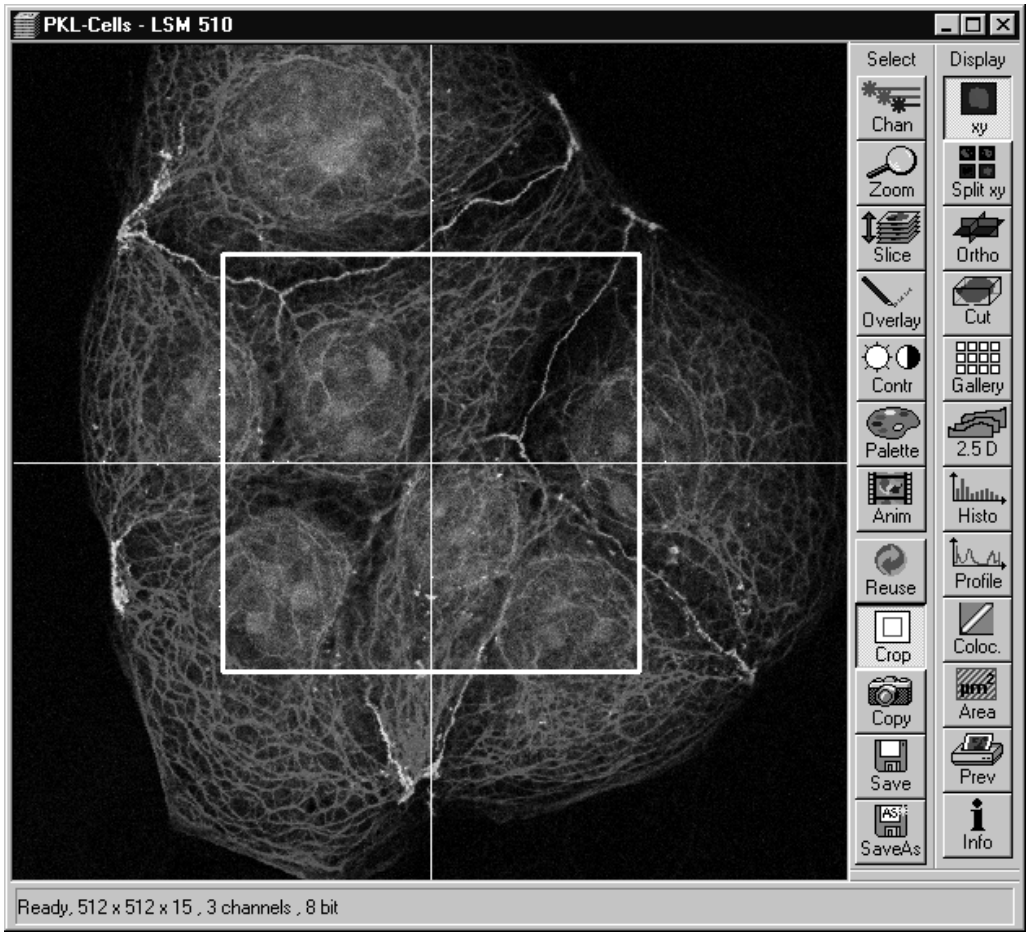


Fig. 4-117

(10) Select - Copy

Copies the image content to the clipboard, from where the image can be accessed and copied to other programs/applications (e.g. the MS-WORD word processor).

(11) Select - Save

Allows you to save the scanned image(s) under the existing name.

(12) Select - Save As

Allows you to save the scanned image(s) under a freely selectable name.

(13) Display - xy

Allows you to switch back to the original image from a different presentation mode, such as Split-Mode or Gallery.

(14) Display - Split xy

In multiple-channel scanning, the image is seen to be generated in the different channels, together with the composite image.

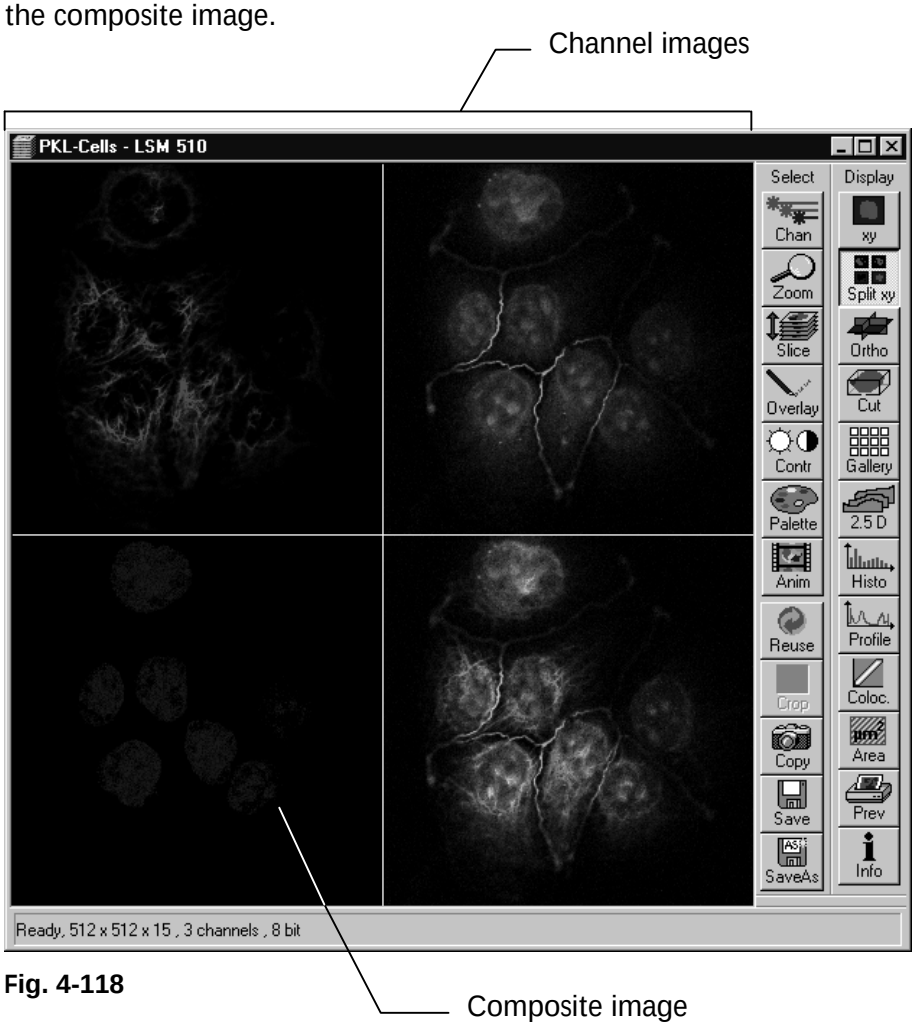


Fig. 4-118

☞ The Display-Split function is helpful in optimizing the various channels in image acquisition.

(15) Display - Ortho

Orthogonal sections can be made anywhere in a stack. Furthermore, it is possible to measure spatial distances.

Clicking on "Display-Ortho" makes section lines and the correlated section projections in the image appear. On the right, another menu panel entitled **Orthogonal Section** is shown.

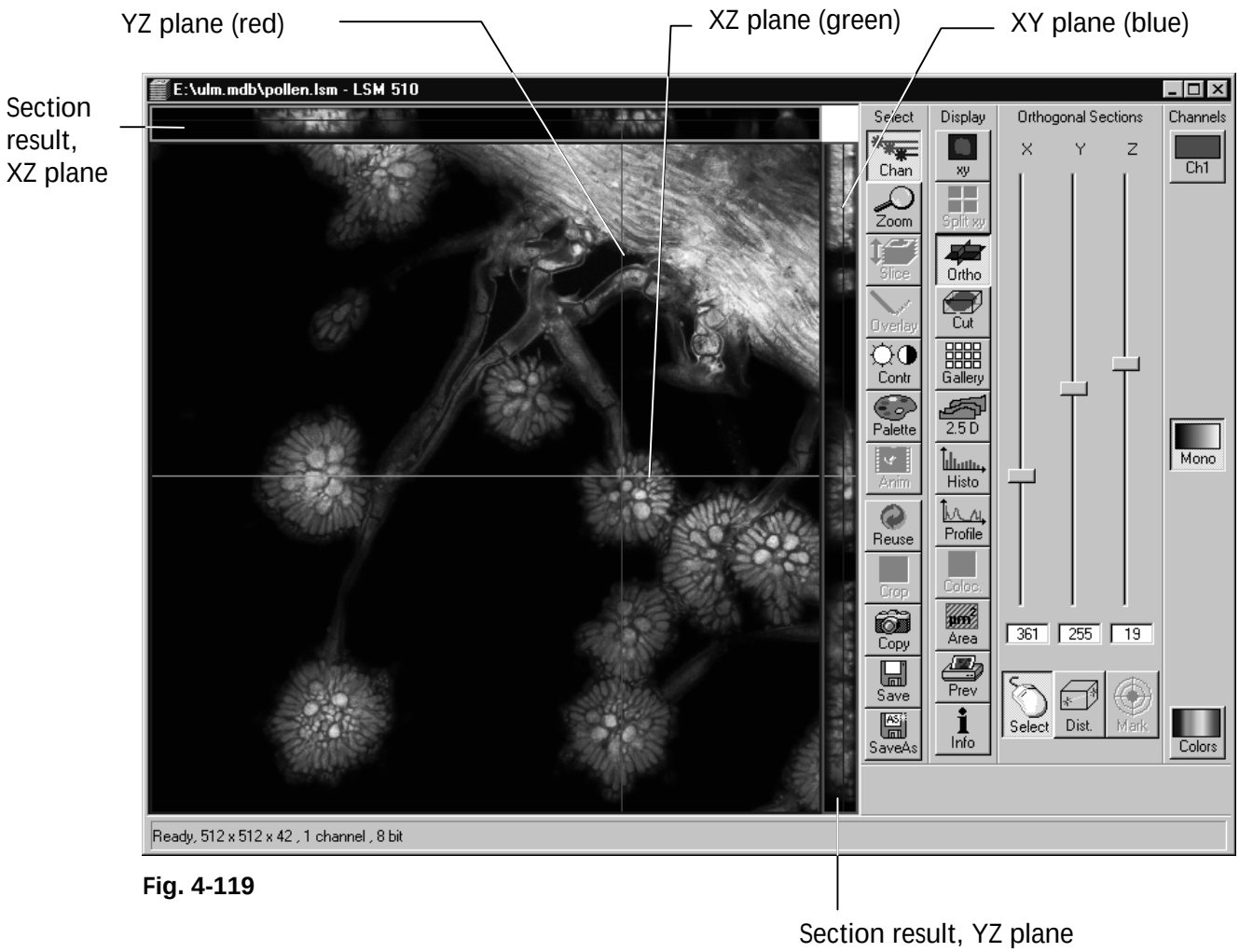


Fig. 4-119

By changing the parameters X, Y and Z on the **Orthogonal Sections** panel, the section plane can be positioned at liberty within the specimen.

The position of the section plane is shown by colored lines.

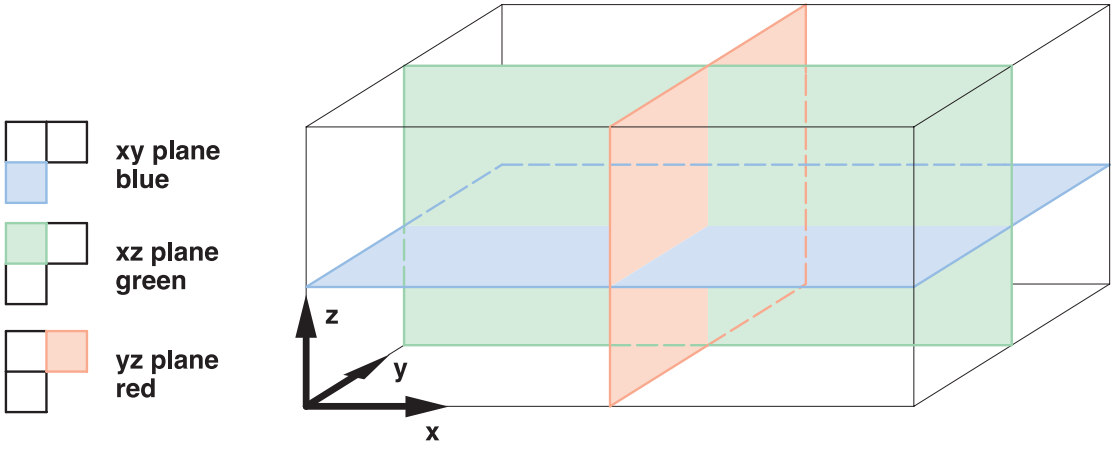


Fig. 4-120

☞ The XY plane (blue) corresponds to the slice planes of the stack.

The position of section planes can be changed in various ways:

- By shifting the sliders on the **Orthogonal Sections** panel.

  X and Y settings may range from 1 up to the maximum number of pixels scanned (in the example shown: 512).

  Z settings may range from 1 to a maximum of n, with n standing for the number of slices in the stack produced.

- If you move the cursor into the image window, it changes into a crosshairs symbol ⊕. By dragging this symbol with the mouse you can position the XZ and YZ section planes to any point of intersection with the XY plane. A click with the left mouse button places the intersection to the desired position.
- If you move the crosshair symbol ⊕ onto the intersection of the red and green section planes, it changes into the: ✣ symbol. If you now press the left mouse button and keep it pressed you can reposition both section planes **at a time**.
- If you move the crosshairs symbol ⊕ onto the green section plane, it changes into the ↕ symbol. If you now press the left mouse button and keep it pressed, you can reposition the (green) XZ section plane.

- You can reposition the (red) YZ plane in the same way using the ↔ symbol.

The result of an orthogonal section is visible at the image margin, no matter which method you used.

- Section of the XZ plane (green) through the stack: above the image.
- Section of the YZ plane (red) through the stack: right of the image.

☞ Activating the <Dist.> button makes length measurements of spatial diagonals possible. The length of the yellow measuring line is shown in µm below the button, e.g. 3D Distance: 52.00 µm

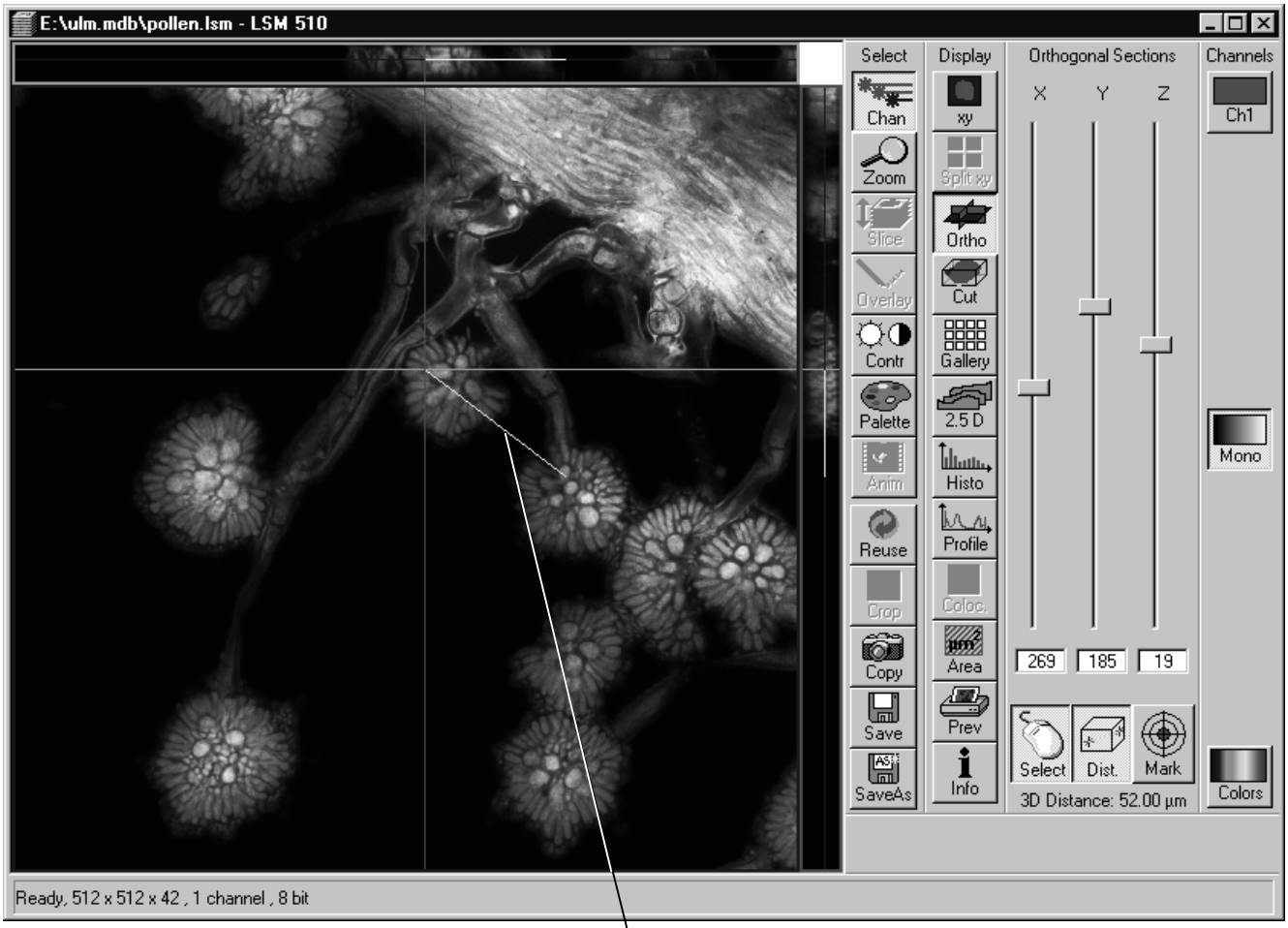


Fig. 4-121

(16) Display - Cut

In a stack you can generate sections along a plane of freely selectable positions.

Clicking on the <Display-Cut> button opens the **Cut** dialog panel to the right of the image processing toolbar.

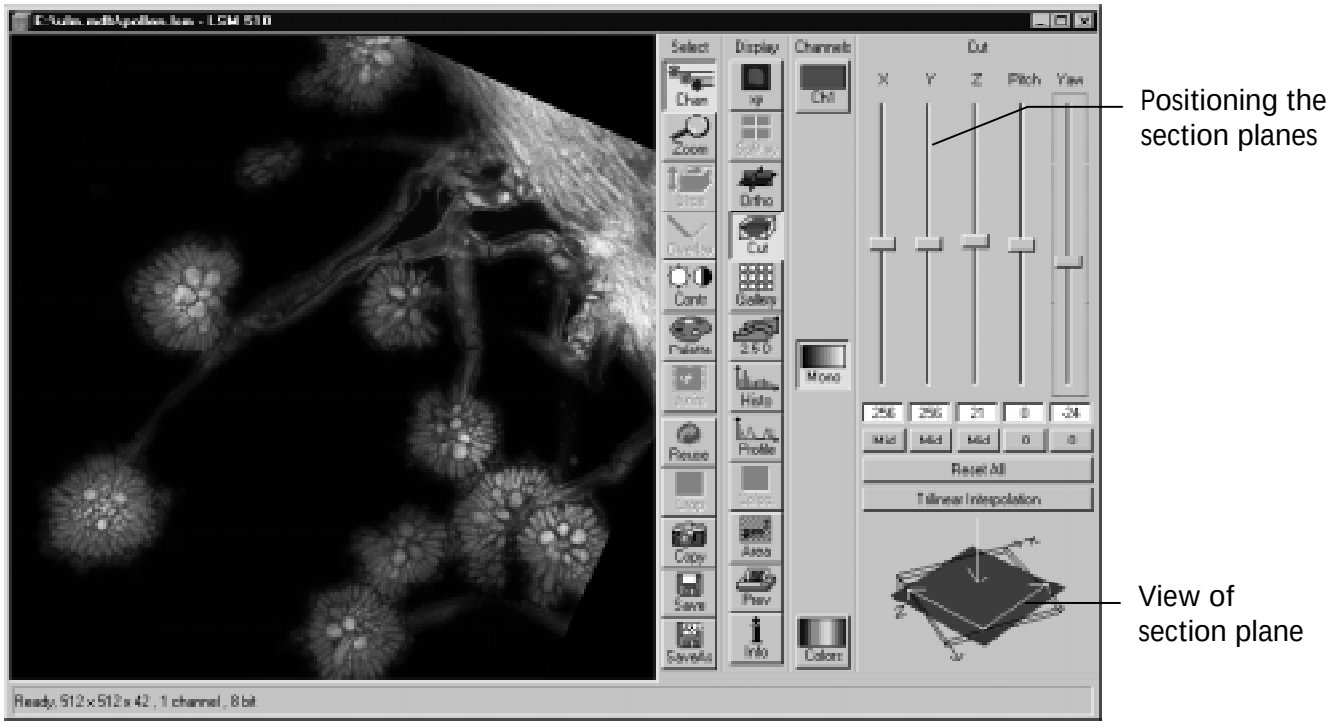


Fig. 4-122

By varying the parameters X, Y, Z, Pitch and Yaw, you can position a section plane of any inclination, with any point within the stack volume as the datum.

The resulting position of the section plane is shown as a red area below the <Trilinear Interpolation> button. At the same time, the result is shown in the image window.

A click on the <Reset All> button restores the original position.

(17) Display - Gallery

The various slices of a stack are shown in a tiled presentation of several rows (called a gallery), in chronological order of their generation.

A click on the <Display-Gallery> button not only produces the gallery itself but also the **Gallery** panel with two buttons: <Data> button and <Subset> button.

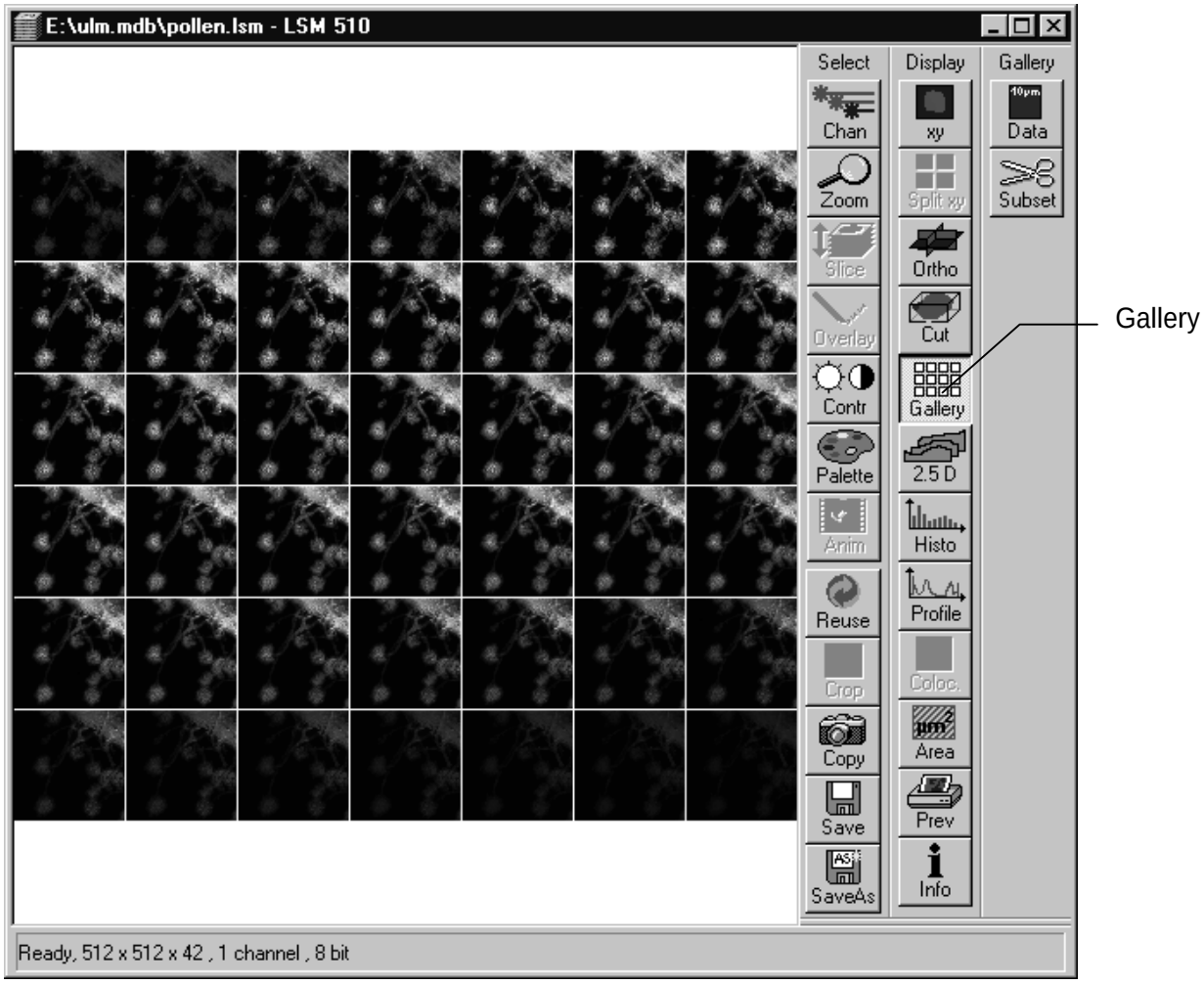


Fig. 4-123

Clicking on the <Data> button causes the Z data to be entered into the images of the stack. This provides you with information on the Z distance of each section (slice) relative to the first section plane. In a time series, the temporal interval is shown in seconds.

Clicking on the <Subset> button opens another window entitled "Subset", in which you can select certain images of the stack.

The system then generates and displays a stack consisting of the selected images only.

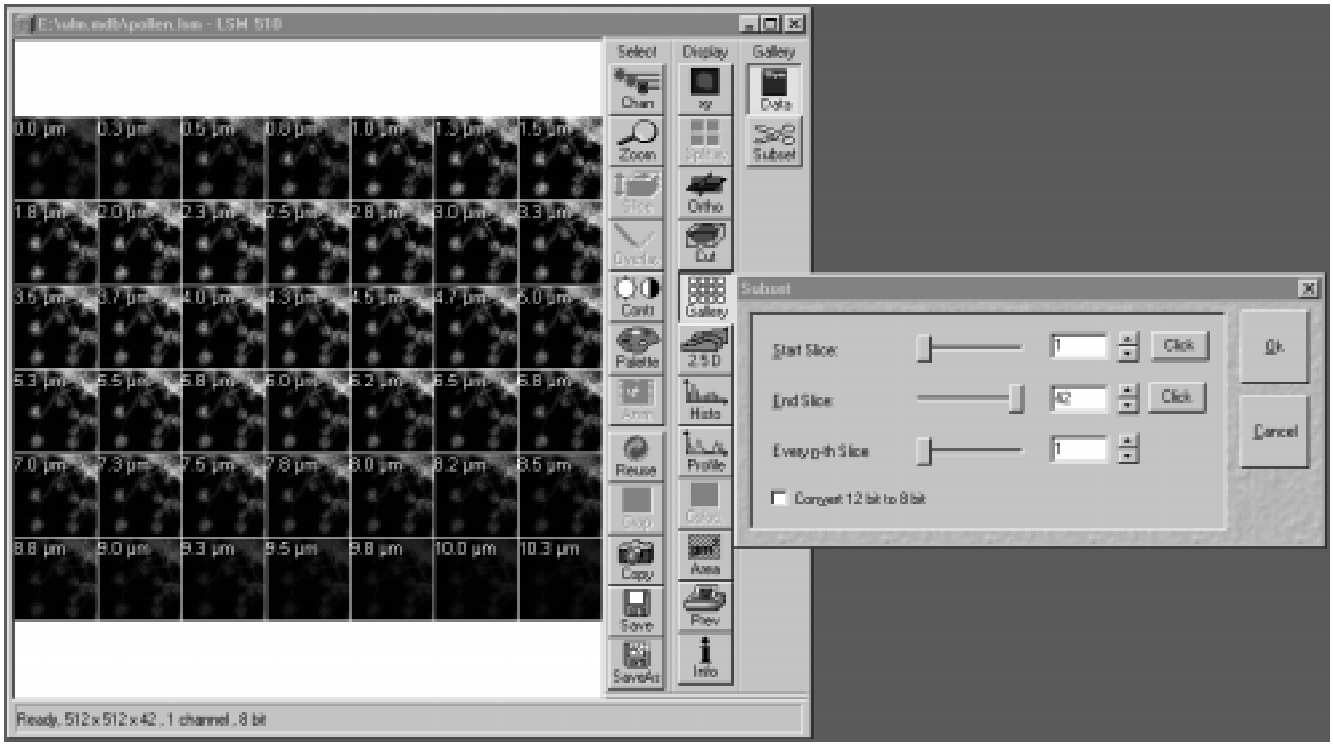


Fig. 4-124

(18) Display - 2.5D

Clicking on this button displays an image in a pseudo-3D mode, representing the intensity distribution over the scanned area, and opens a panel headed "Pseudo 3D", in which you can select various presentations with the <Profile>, <Grid> and <Filled> buttons.

Via the <Filled> button you can select between monochrome and color presentations.

In the "Channel" list box you can select the desired channel.

The scroll bars on the right of the image window permit the viewing plane to be rotated and tilted and the intensity scale to be varied.

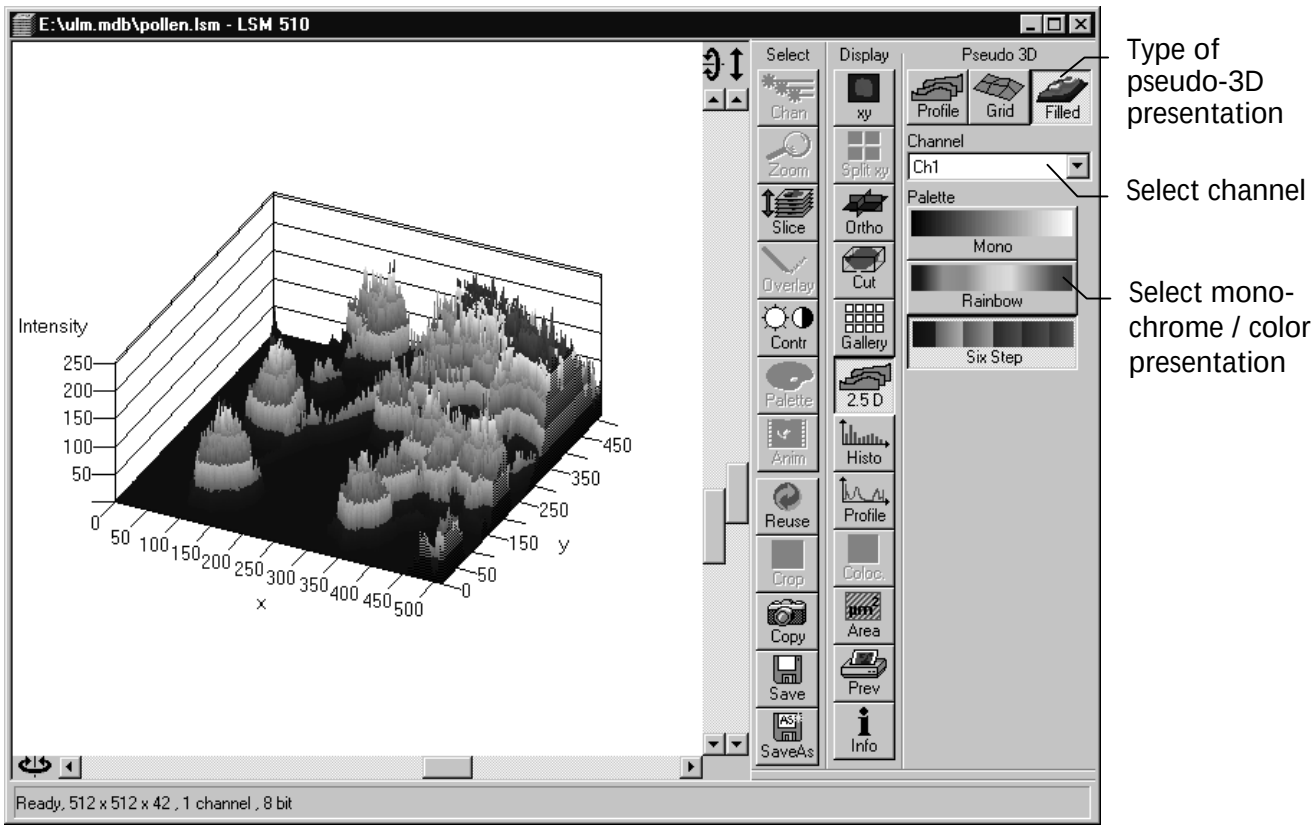
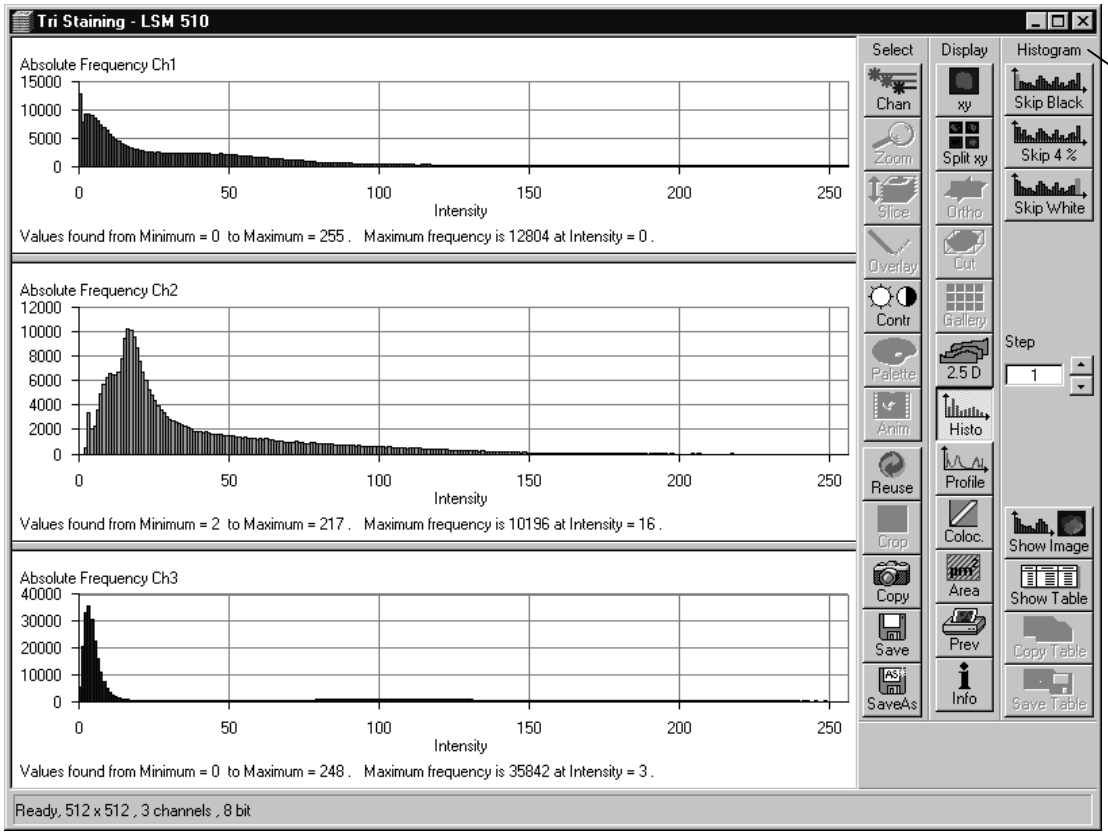


Fig. 4-125

(19) Display - Histo

The histogram function displays a graph of the intensity distribution of an image as well as information on the frequency of the various intensities, separate for each channel.



Control of histogram presentation

Fig. 4-126

(20) Display - Profile

The Display-Profile function shows the intensity profile across the image along a freely selectable line. In multiple-channel mode, the intensity profile is shown separately for each channel.

The intensity curves are shown in a graph below the scanned image.

On the **Profile** panel you can select the width and color of the cutline.

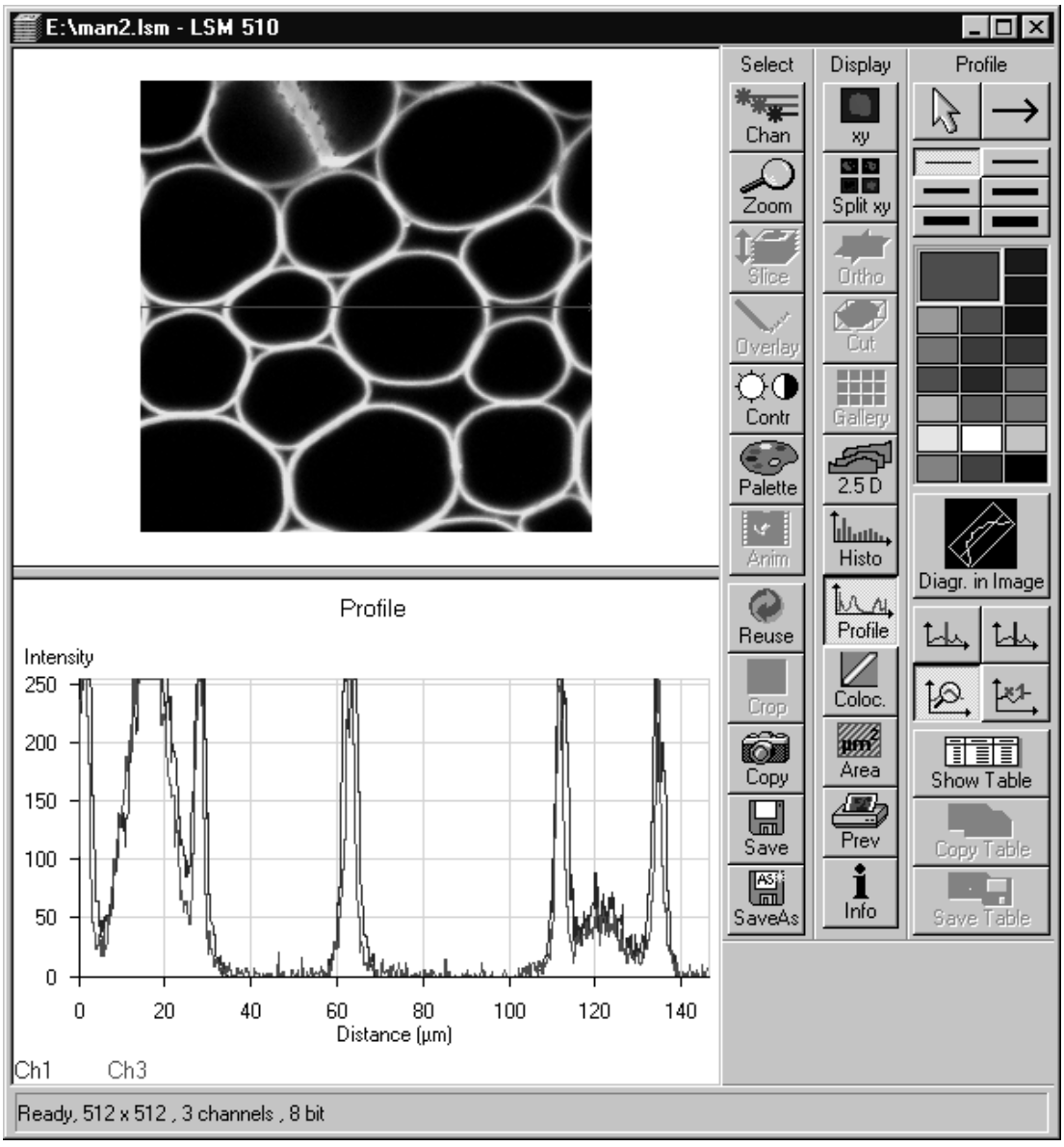


Fig. 4-127

You can place markings wherever you like and follow up this line detecting the intensity profile.

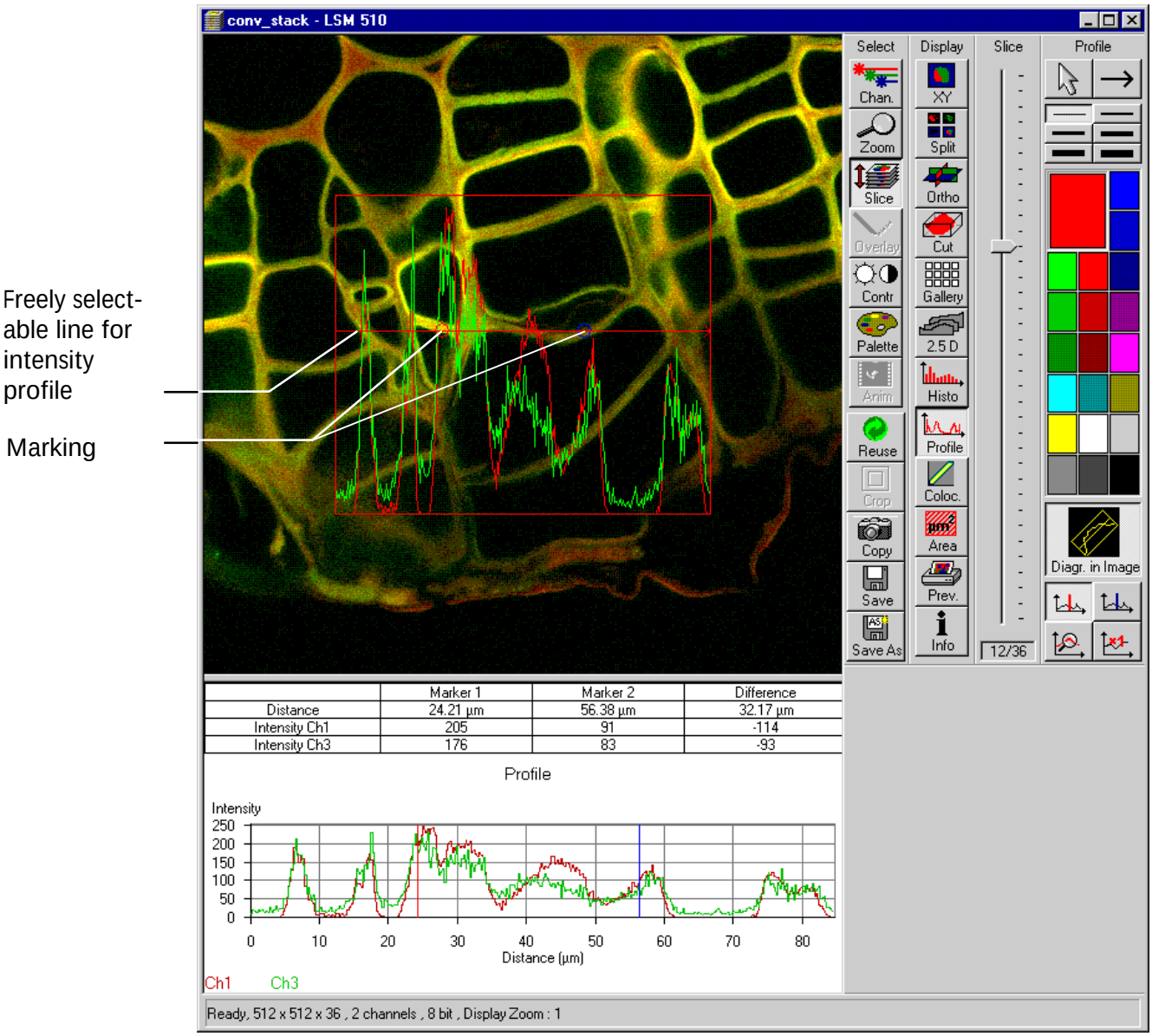


Fig. 4-128

Click on the <Diagr. in Image> button to overlay an intensity graph directly on the image.

(21) Display - Coloc.

The Display-Coloc. function presents a comparison between two images by computing a scatter diagram (colocalization).

How a scatter diagram is generated:
All pixels having the same positions in both images are considered a pair. Of every pair of pixels (P1, P2) from the two source images, the brightness level of pixel P1 is interpreted as X coordinate, and that of pixel P2 as Y coordinate of the scatter diagram. The value of the pixel thus addressed is increased by one every time, up to the maximum number of pixels used. This way, each pixel of the scatter diagram is a value that shows how often a particular pair of pixels has occurred.

Differences between the images cause irregular spots in the scatter diagram.

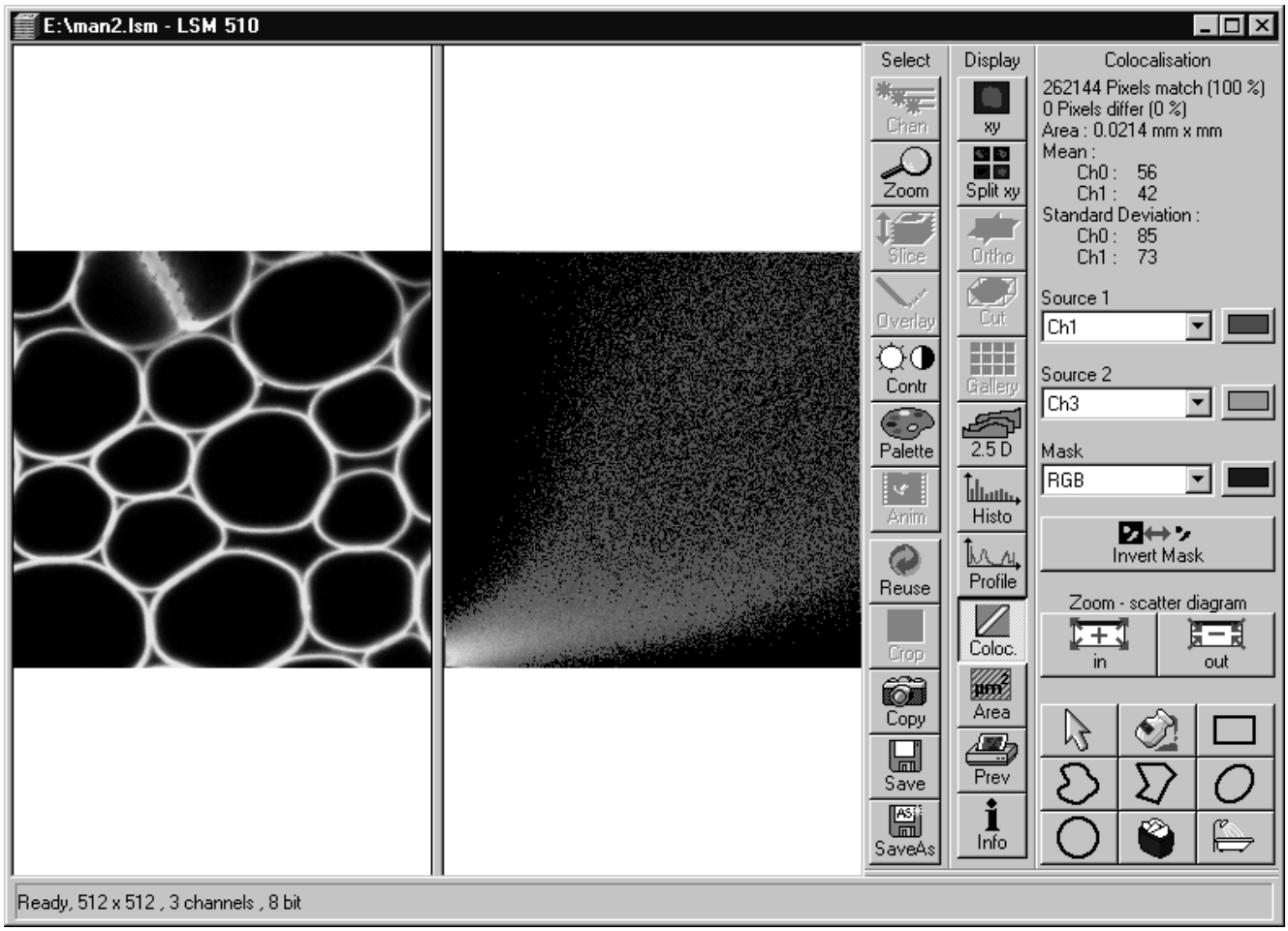


Fig. 4-129

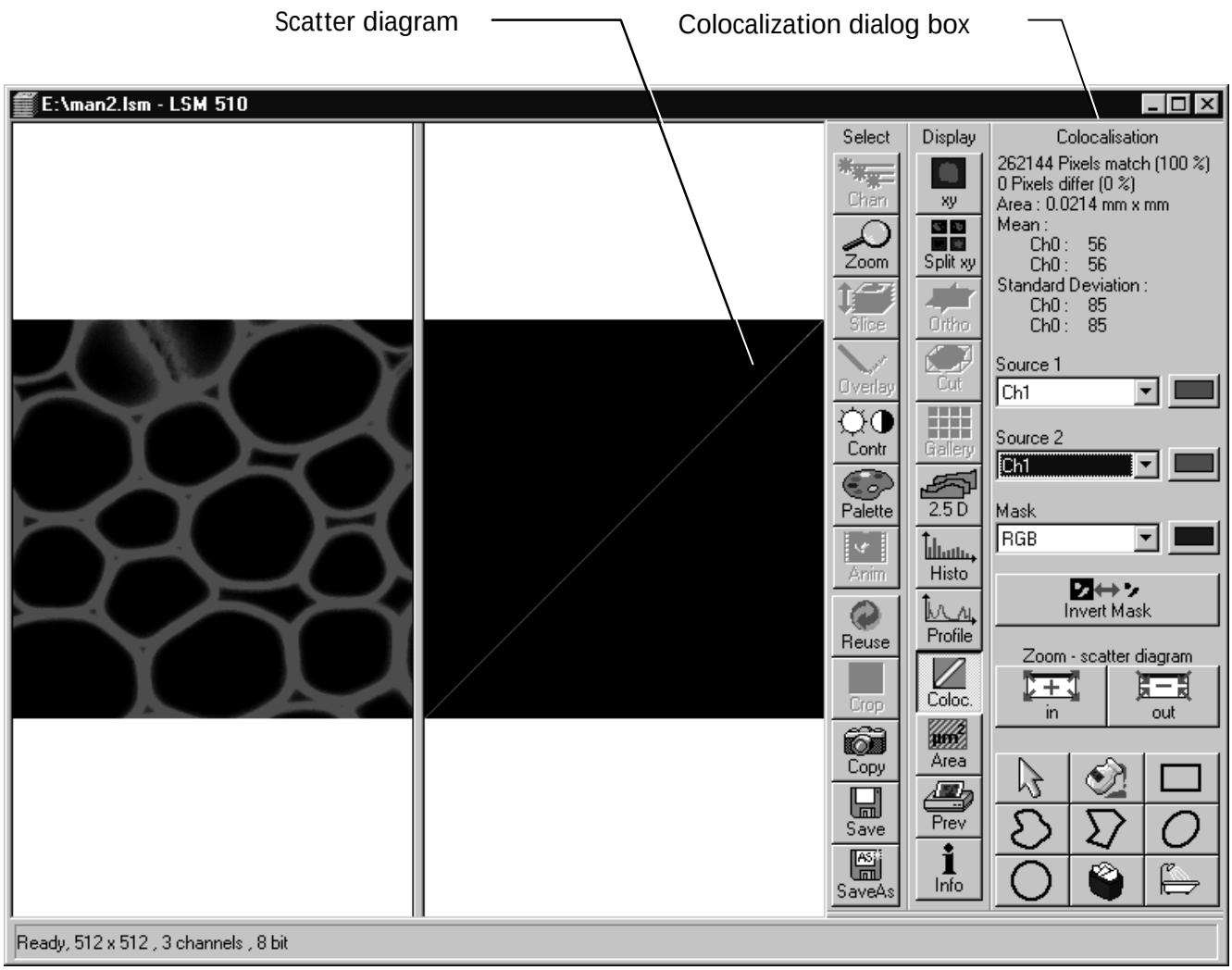


Fig. 4-130

Identical images produce a clean diagonal line running from bottom left to top right, because only pixel pairs (0,0), (1,1), (2,2) etc. can occur. Differences between the images cause irregular spots in the scatter diagram.

(22) Display - Area

Clicking on the <Display-Area> button opens the **Area Measure** dialog box. The top area of this panel shows the geometric size of the scanned image.

This function allows you to measure the area of any plane geometric figure within the scan image. The function can be activated by clicking on one of the geometry buttons, e.g. (polyline). The figure of interest can be marked in the image by cursor control in conjunction with a mouse click.

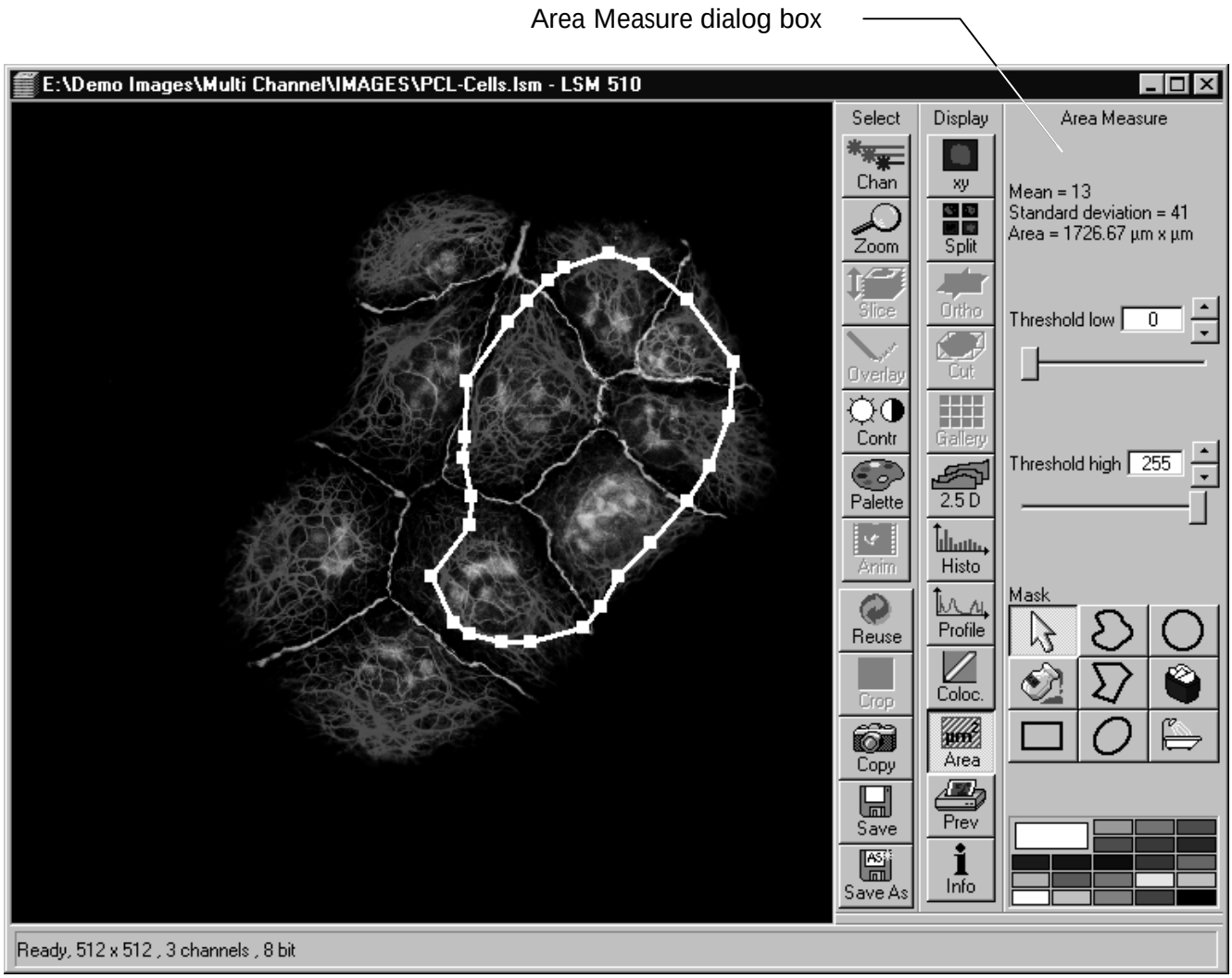


Fig. 4-131

Clicking on the <Flood fill> button (paint jar) and moving the cursor to the area to be excluded causes the remaining area to be computed and the result indicated under Area Measure.

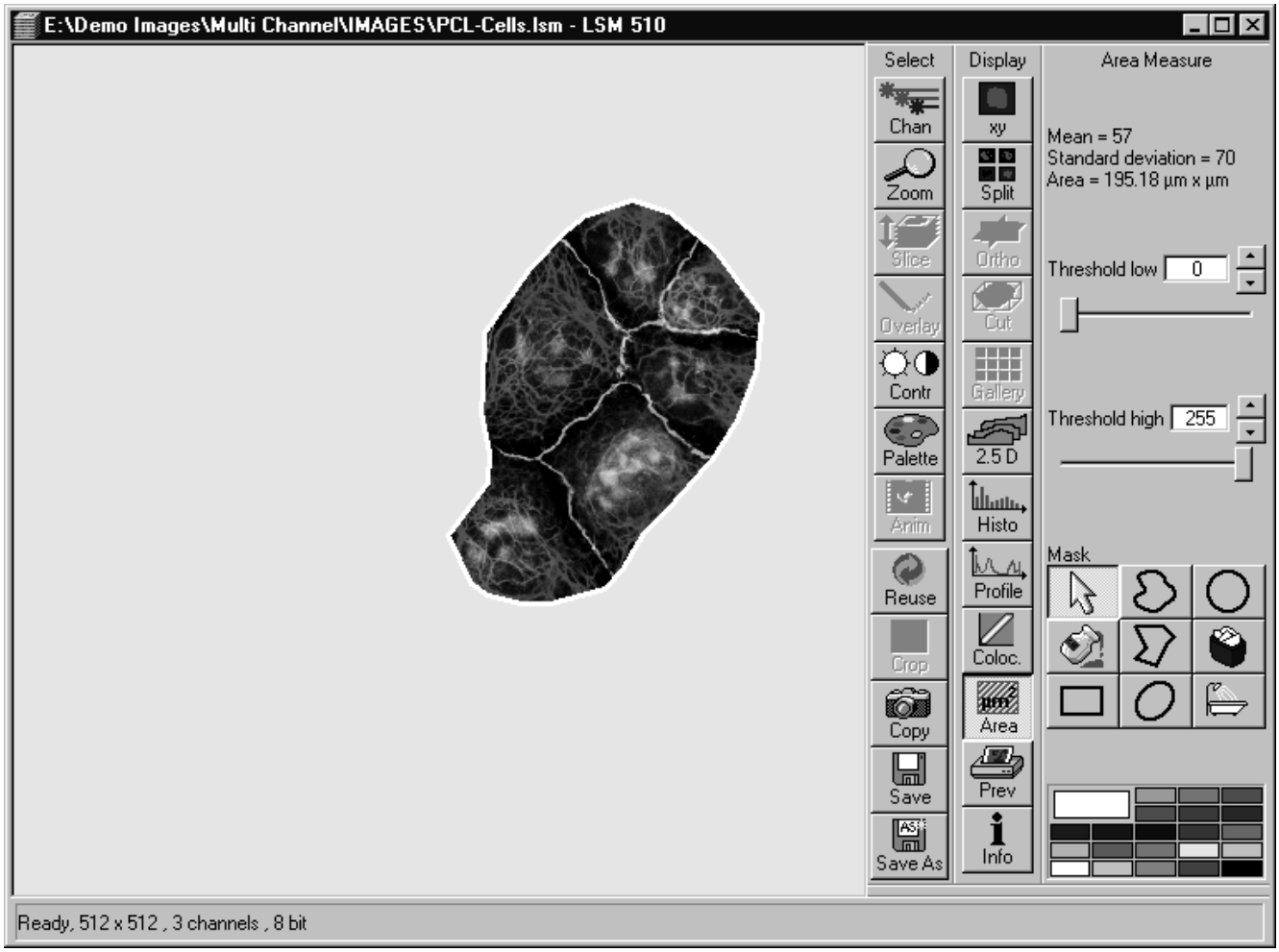


Fig. 4-132

If you specify a top and bottom intensity threshold, the area lying within this intensity interval can be computed.

Specify the thresholds either with the Threshold low and Threshold high sliders, or with the and buttons.

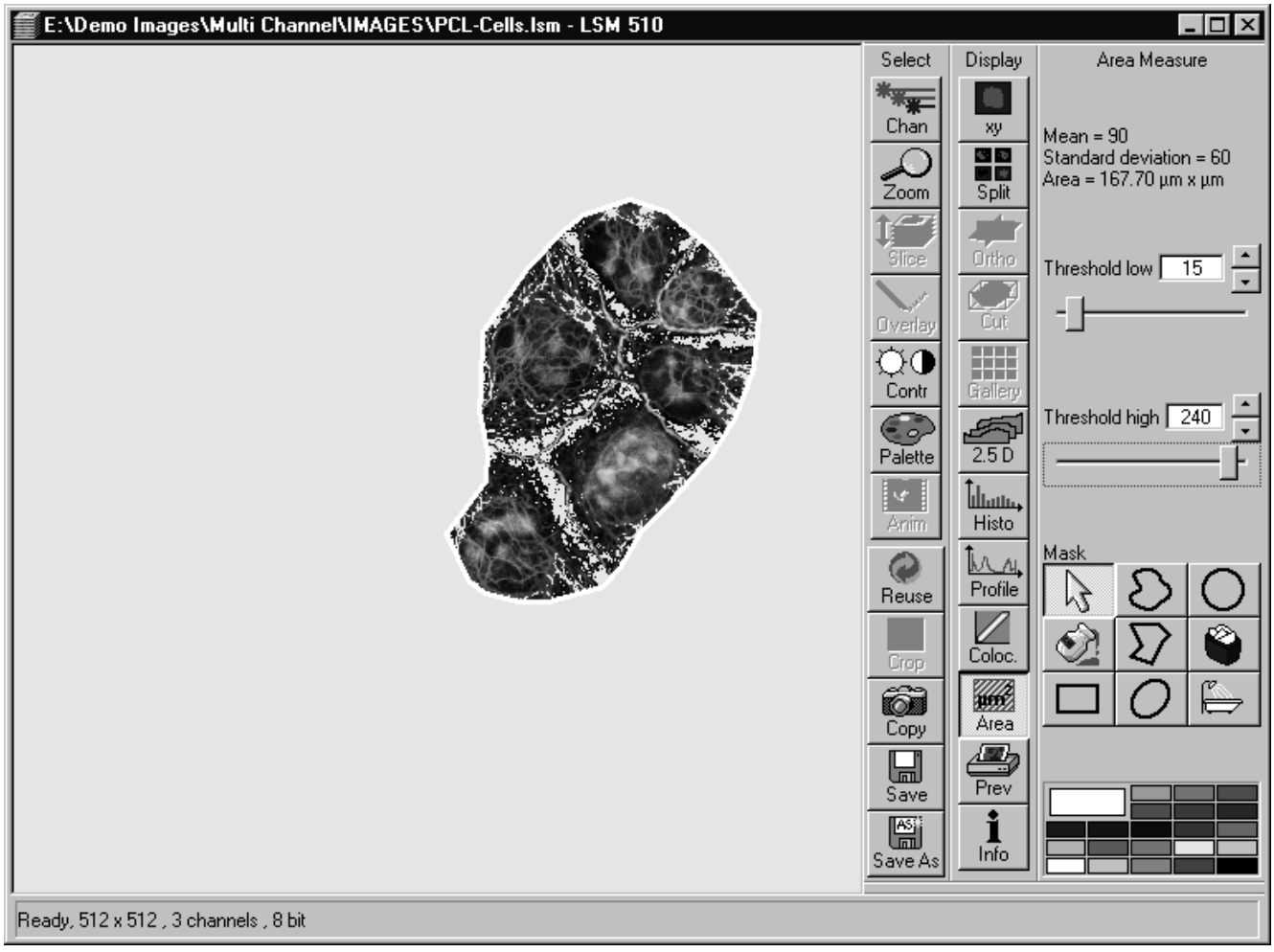


Fig. 4-133

Click on the <Display> button if you want to return to the original image.

(23) Display - Prev.

This function enables you to assemble a preview of all pictorial, textual and graph information you want to print out.

The size and position of the image can be varied using the mouse pointer in the image window.

Click on the <Info> button of the image processing toolbar if you want to view print status information and include it in the print preview.

☞ The information shown is limited to that previously specified via Options/Settings/Register Print Status Display.

Click on the <Arrange> button for optimum layout of image size and position relative to the textual information.

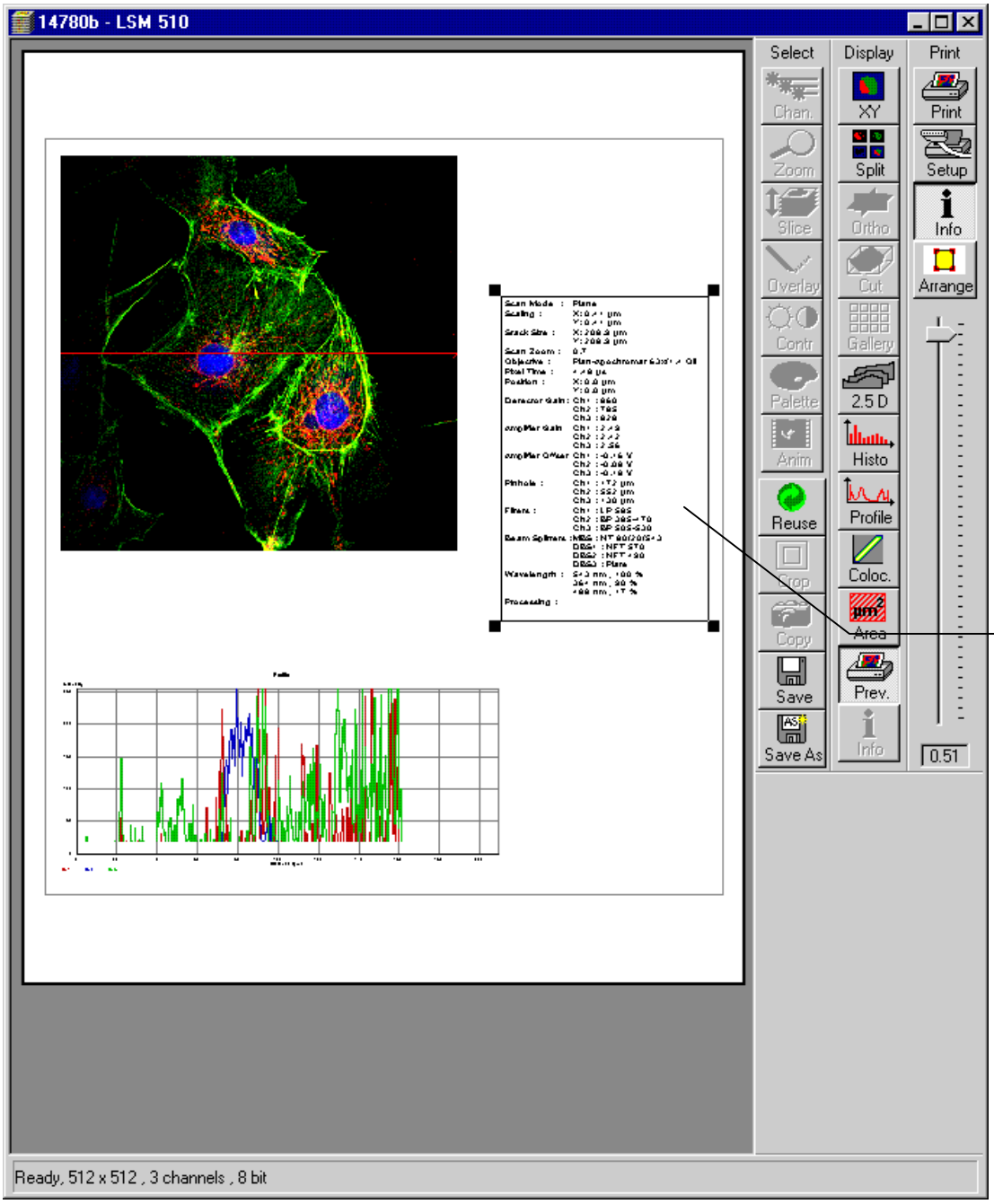

14780b - LSM 510
Select
Display
Print
Chan.
XY
Print
Zoom
Split
Setup
Slice
Ortho
Info
Overlay
Cut
Arrange
Contr
Gallery
Palette
2.5 D
Anim
Histo
Reuse
Profile
Crop
Coloc.
Copy
Area
Save
Prev.
Save As
Info
0.51
Ready, 512 x 512 , 3 channels , 8 bit
Assembly of image, intensity profile and scan info

Fig. 4-134

A layout generated with **Prev.** (Preview) can be printed by clicking on the <Print-Print> button.

Clicking on the <Print-Setup> button opens the "Print Setup" window, in which you can specify print parameters.

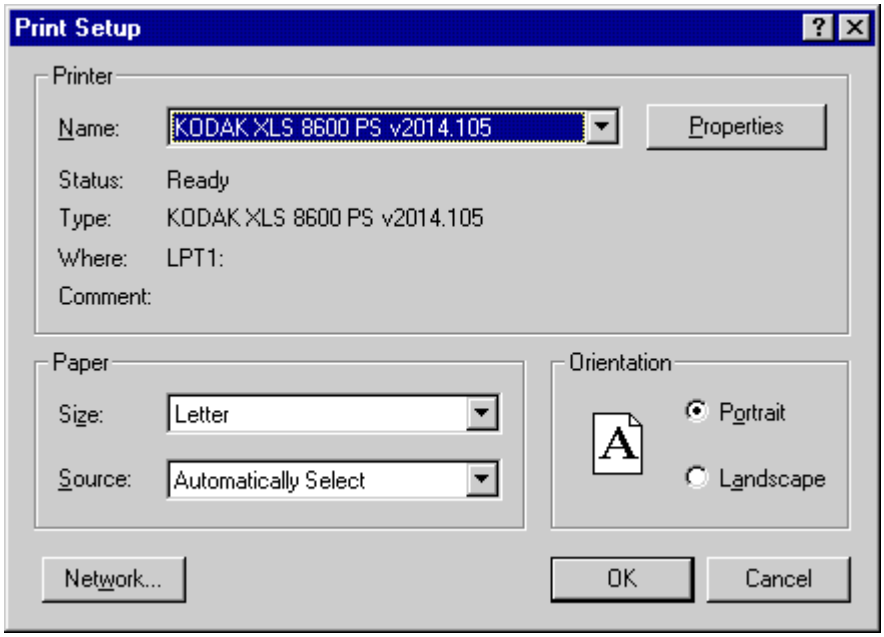


Fig. 4-135

(24) Display - Info

Clicking on the <Info> button, all parameters used to generate the image appear at the left image side.

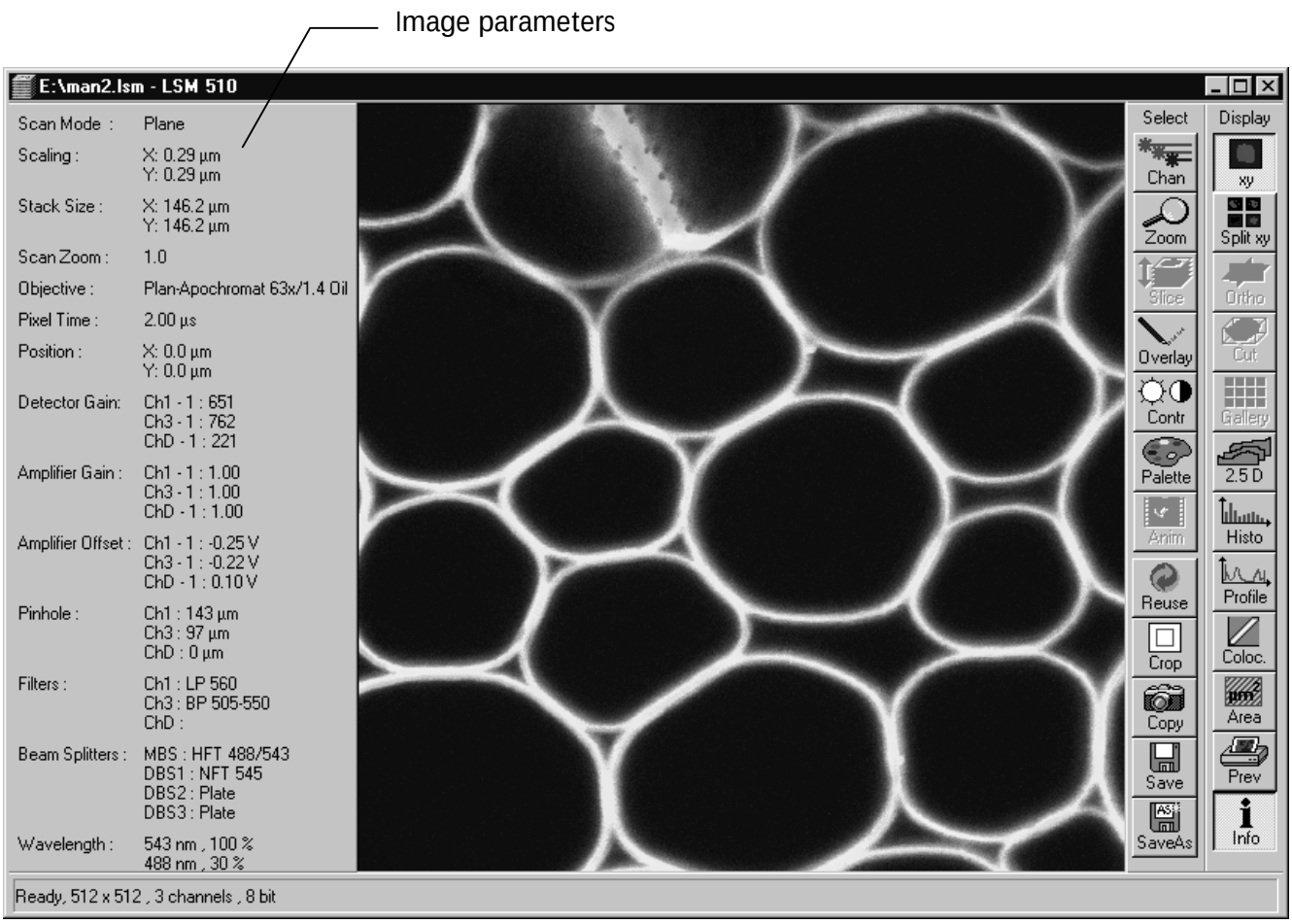

Image parameters
E:\man2.lsm - LSM 510
Scan Mode : Plane
Scaling : X: 0.29 µm Y: 0.29 µm
Stack Size : X: 146.2 µm Y: 146.2 µm
Scan Zoom : 1.0
Objective : Plan-Apochromat 63x/1.4 Oil
Pixel Time : 2.00 µs
Position : X: 0.0 µm Y: 0.0 µm
Detector Gain: Ch1 - 1 : 651 Ch3 - 1 : 762 ChD - 1 : 221
Amplifier Gain : Ch1 - 1 : 1.00 Ch3 - 1 : 1.00 ChD - 1 : 1.00
Amplifier Offset : Ch1 - 1 : -0.25 V Ch3 - 1 : -0.22 V ChD - 1 : 0.10 V
Pinhole : Ch1 : 143 µm Ch3 : 97 µm ChD : 0 µm
Filters : Ch1 : LP 560 Ch3 : BP 505-550 ChD :
Beam Splitters : MBS : HFT 488/543 DBS1 : NFT 545 DBS2 : Plate DBS3 : Plate
Wavelength : 543 nm , 100 % 488 nm , 30 %
Select
Chan
Zoom
Slice
Overlay
Contr
Palette
Anim
Reuse
Crop
Copy
Save
SaveAs
Display
xy
Split xy
Ortho
Cut
Gallery
2.5 D
Histo
Profile
Coloc.
Area
Prev
Info
Ready, 512 x 512 , 3 channels , 8 bit

Fig. 4-136

(25) 3D View

- For the further three-dimensional analysis of image sequences (stacks), click on the <3D View> button in the main menu.
  - This opens a submenu bar with the buttons <Depth Cod.>, <Projection> and <Stereo>.

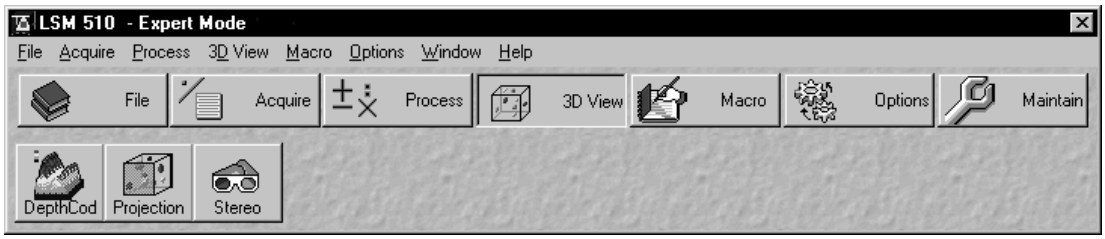


Fig. 4-137

(a) 3D-DepthCod (Color coded depth map)

Requirement:
A stack of images must be available.

Clicking on the <Depth Cod.> button opens the "Depth Coding" window.

The system then generates a color-coded depth map for the selected channel. On the Depth Coding panel you can set the desired parameters. Activate the **Scale Bar** check box if you want a color scale to be shown.

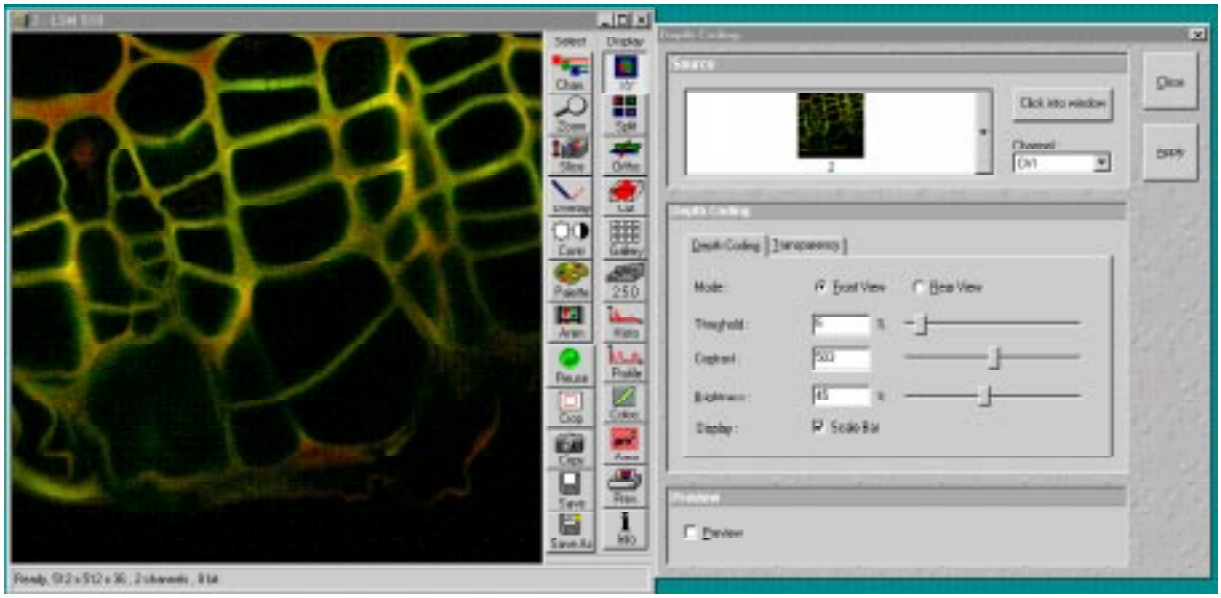


Fig. 4-138

The "Preview" function permits you to regard the influence of parameter changes in an image window.

After finding the optimum adjustment using the "preview" function, you have to generate the final version of the image using the <Apply> button, and the print will be prepared.

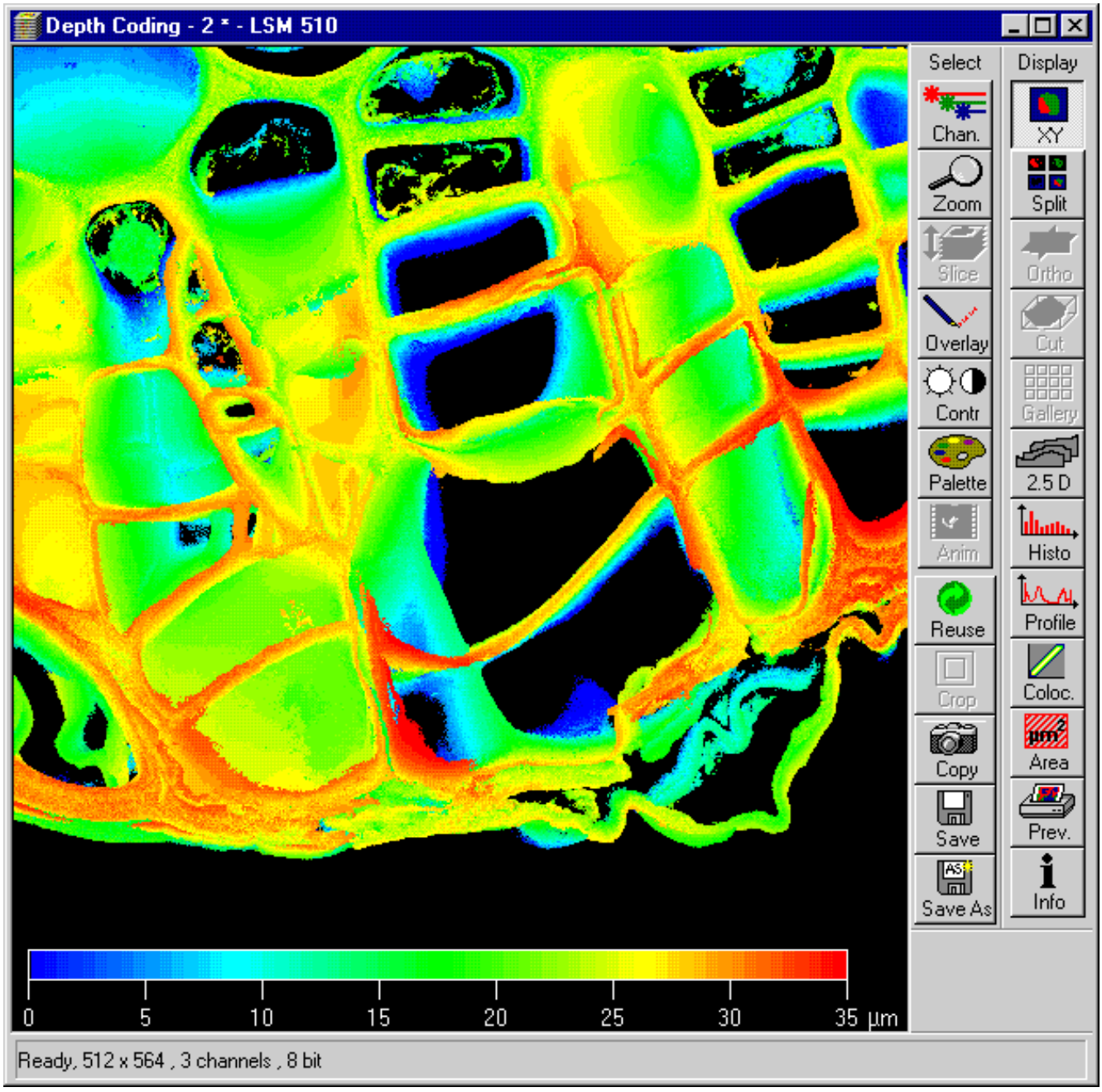


Fig. 4-139

(b) Projection

Requirement:
A stack of images must be available.

Clicking of the <Projection> button opens the "Projection" window.

On the **Projection** panel, set the parameters needed for the animation: Turning Axis, First Angle, Number Projection and Difference Angle.

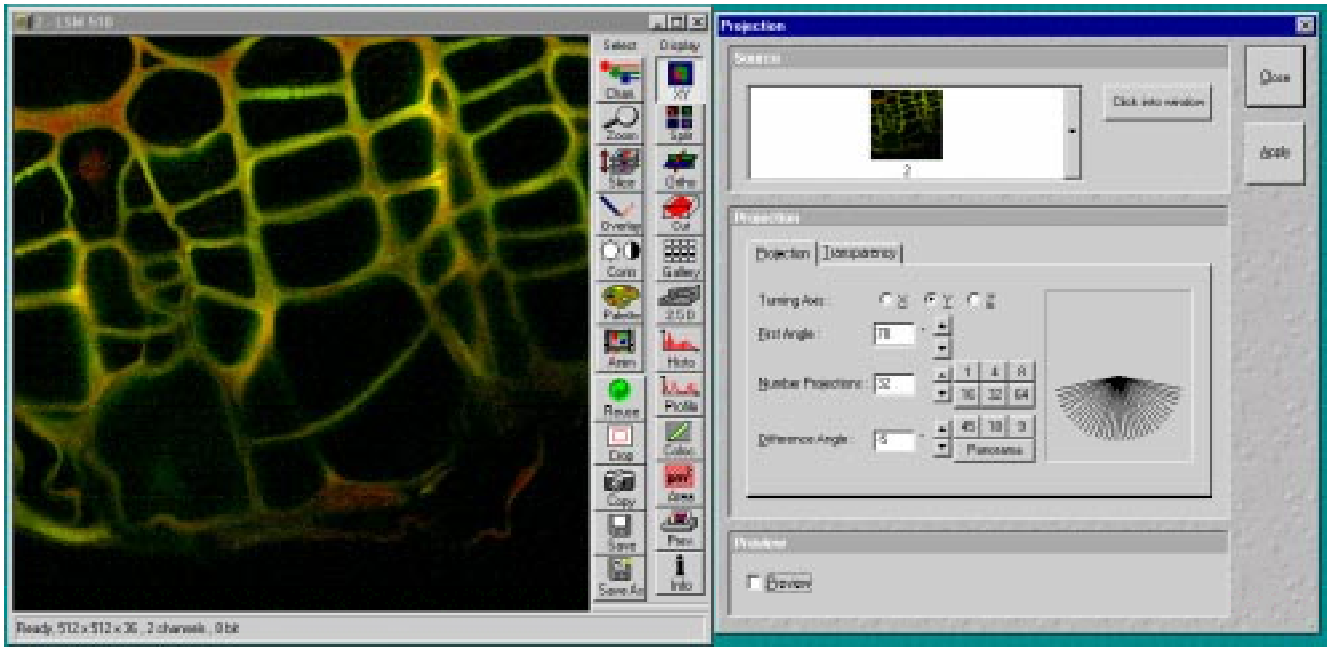


Fig. 4-140

Click on the <Apply> button to have the sequences computed.

– The projection appears. The computation can be followed in the image or by the progress bar.

☞ The computed 3D sequence can be animated with the <Anim> button in the **Select** bar.

– In addition, the "Animate" dialog box appears, in which you can influence the direction and speed of 3D image rotation.

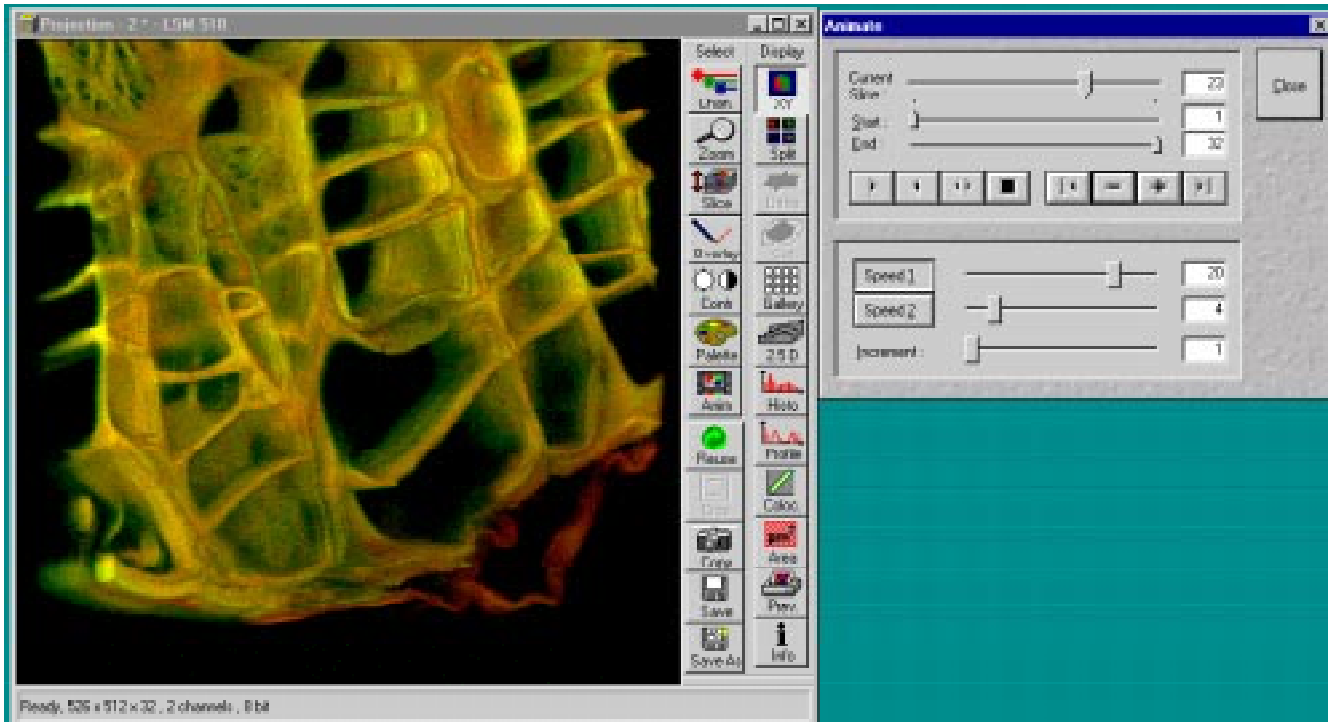


Fig. 4-141

You can browse through the rotation sequence manually with the <Slice> button in the **Select** bar and the "Slice" slider.

☞ To view the computed 3D sequence as a gallery on the screen, click on the <Display-Gallery> button.

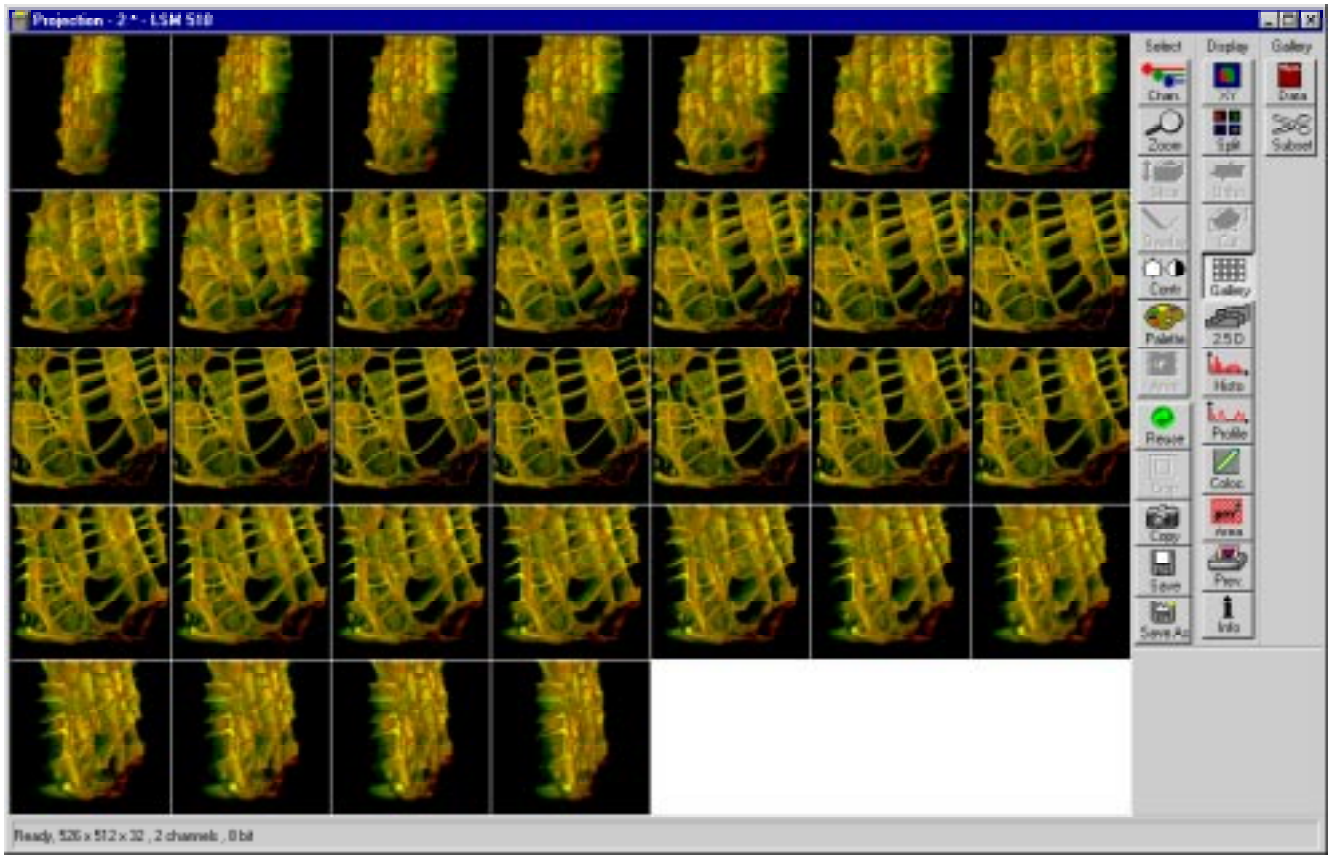


Fig. 4-142

(c) Stereo

Requirement:
A stack of images must be available.

Clicking on the <Stereo> button opens the "Stereo Images" window.

- The image to be processed appears on the **Source** panel.
- On the **Stereo Images** panel, set the parameters needed for stereoscopic viewing, such as

  In the **Projection** register:

  Mode: activate Red/Green Image

  Basic Angle:
  Right Left Angle:
  Number Images:
  Difference Angle:

  ⌐ Enter desired values ⌙

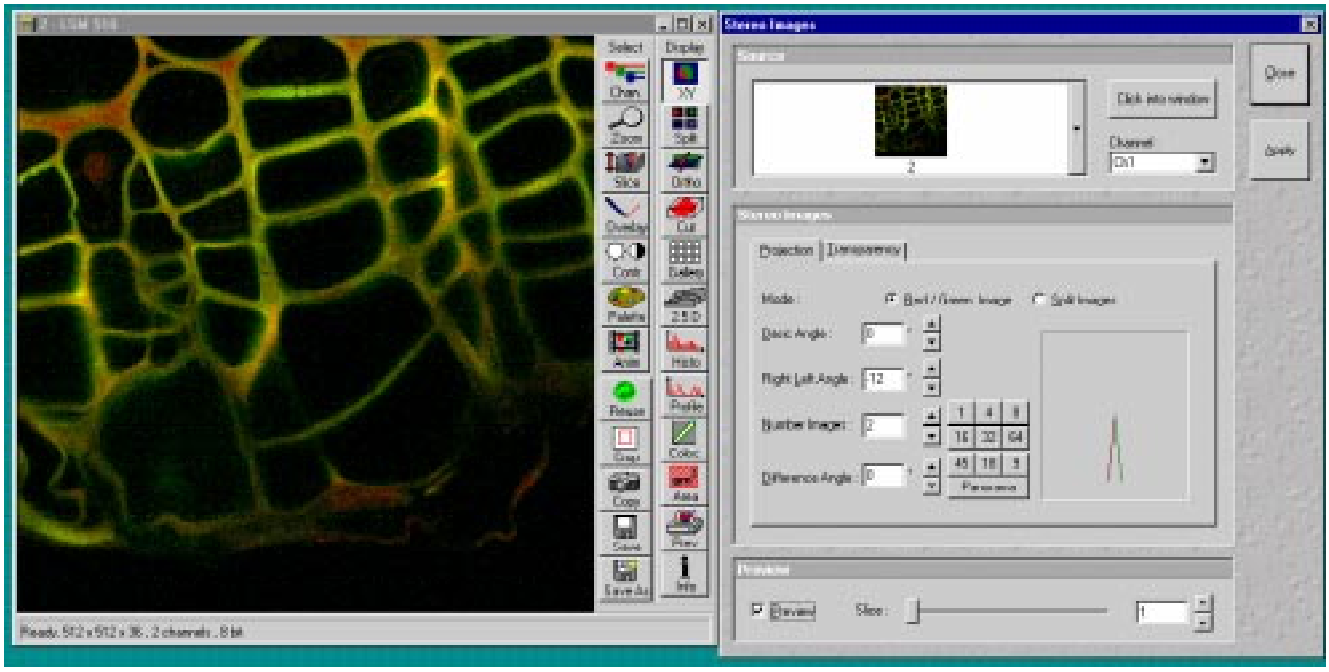


Fig. 4-143

To start computation of the stereoscopic image, click on the <Apply> button.

– The image is built up twice (once each for the red and green colors), resulting in a stereoscopic image.

☞ The stereoscopic effect can only be seen with the aid of red/green 3D goggles.

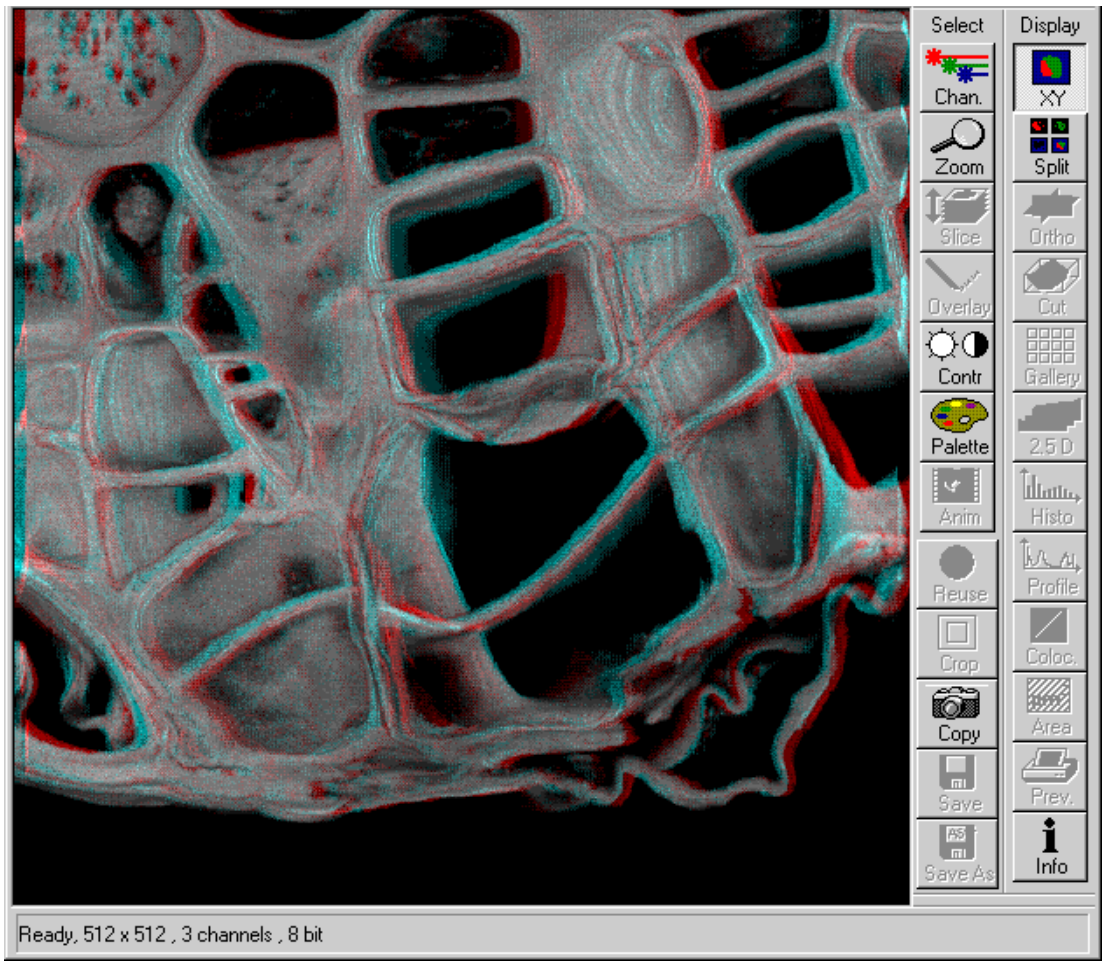


Fig. 4-144

☞ The presentation can be modified by selecting the Split Images function in the Projection register of the **Stereo Images** panel.

On clicking on the <Apply> button, the two stereo mates are presented side by side and can be viewed without red/green 3D goggles.

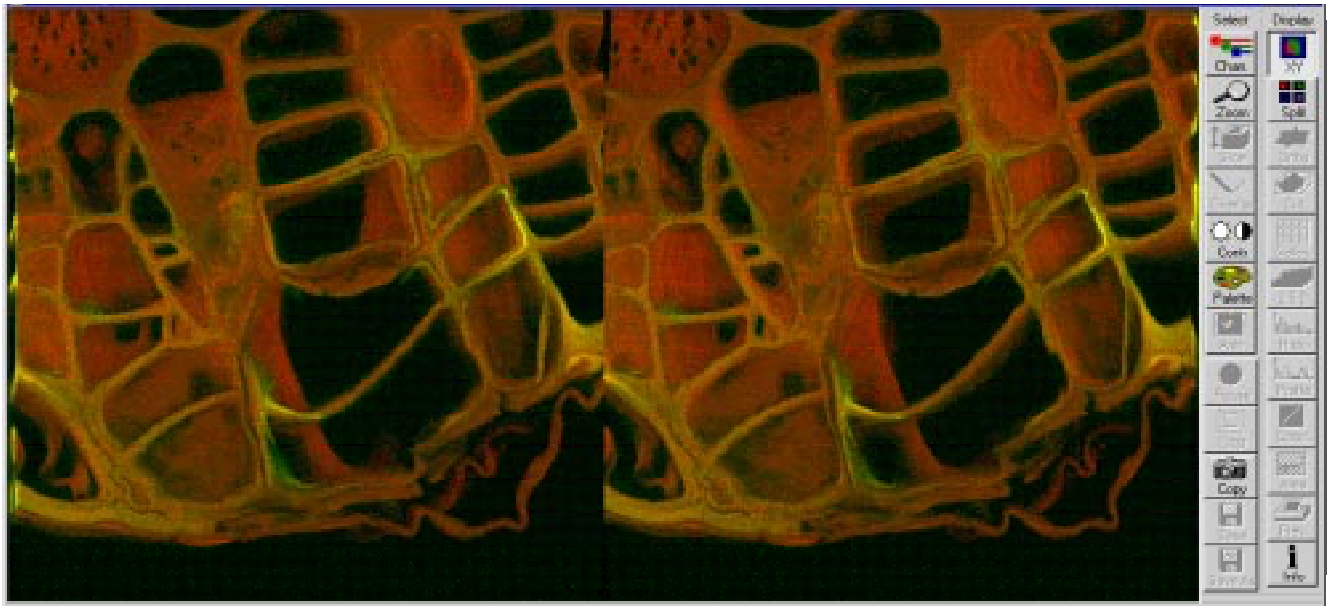


Fig. 4-145

4.9 Data base / Loading and Storing of images

All the generated images are stored in existing or new databases (*.mdb). To load an image, the relevant database must be opened first. Individual images can be loaded using the **Import** function.

4.9.1 Create a new image database

- Click on the <File> button of the main menu toolbar.
  - This opens another subordinate toolbar in the main menu.

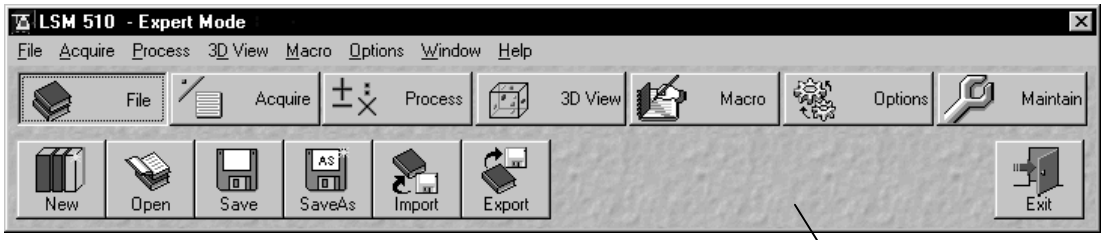


Fig. 4-146

Subordinate toolbar for the File menu item

- Click on the <New> button.
  - This opens the "Create New Database" window for the selection of drives, directories and subdirectories.

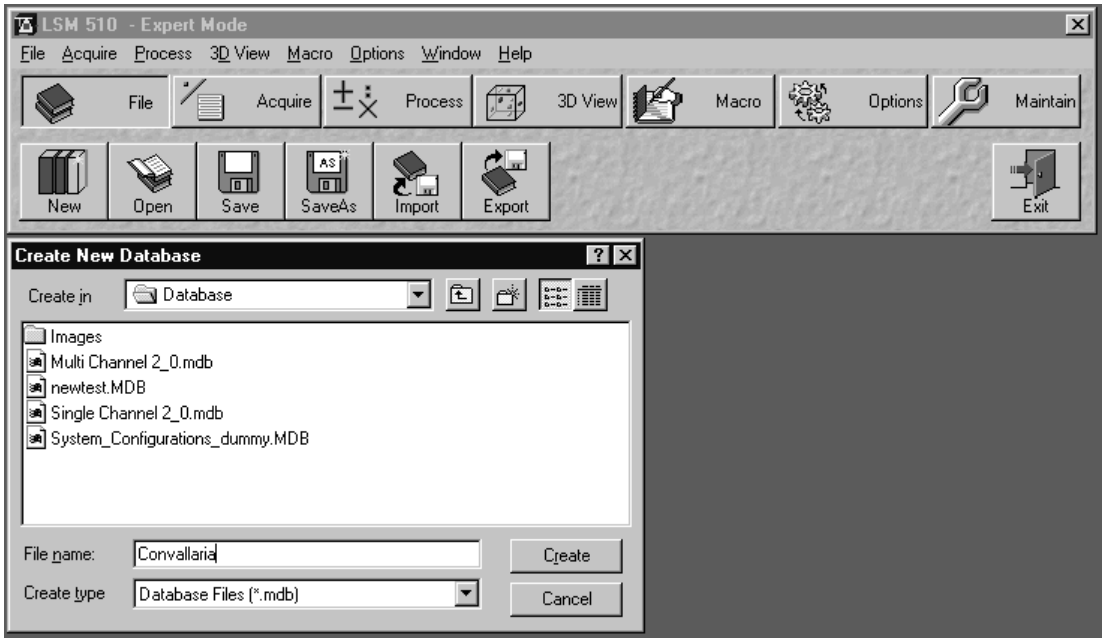


Fig. 4-147

- Enter the name of the database you want to create in the **File name** text box, e. g. "Convallaria".

- If you want to create the image database in a certain folder (drive/directory), click on the arrow button next to the **Create in** box.
  - This opens a drop-down list box showing all folders available for selection.

☞ To look into a superordinate folder, click on the  button.

- Click on the <Create> button.
  - This creates the new image database in the selected drive and directory.

- The "Convallaria.mdb" window appears, presenting the opened database with 0 of 0 image entries.

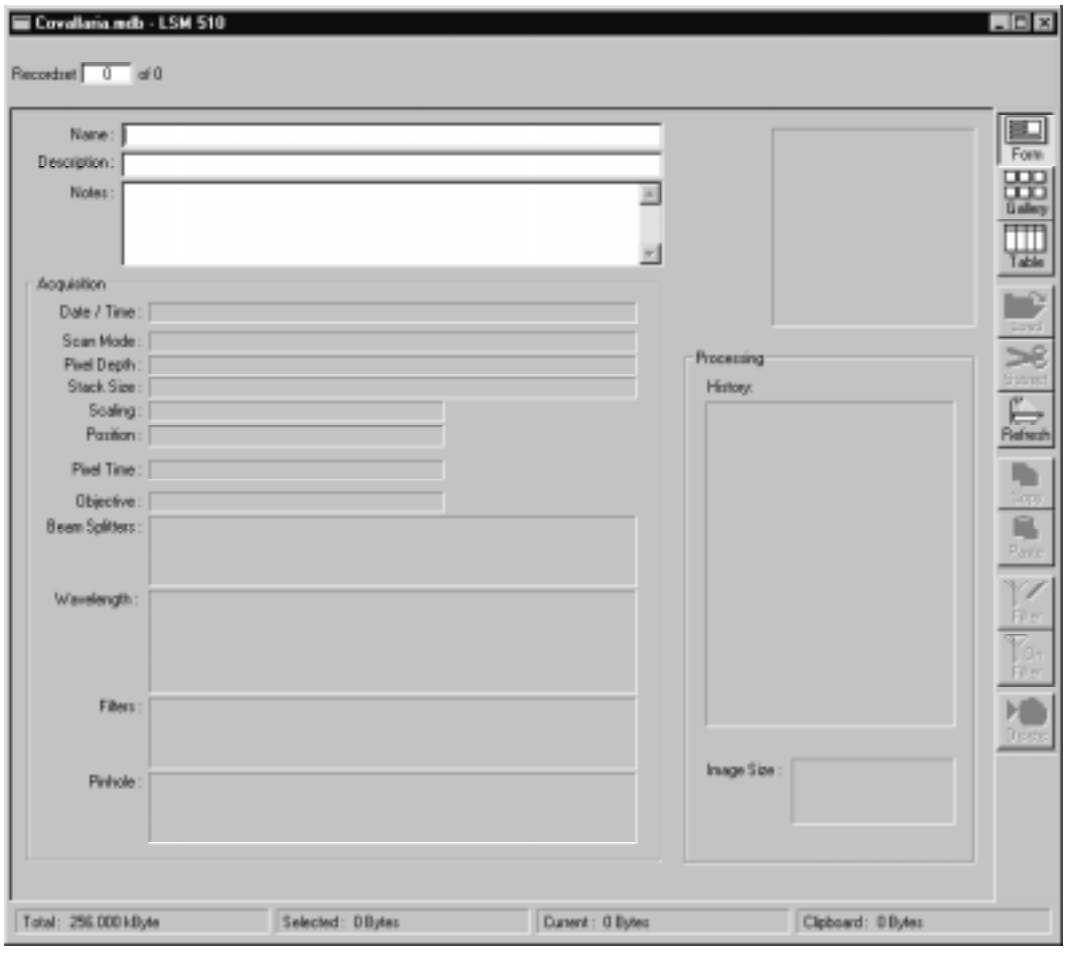


Fig. 4-148

The new image database stores a newly created or changed image (see section 4.11.3).

4.9.2 Loading an image from database

- Click on the <File> button of the main menu toolbar.
  - This opens another, subordinate toolbar in the main menu.

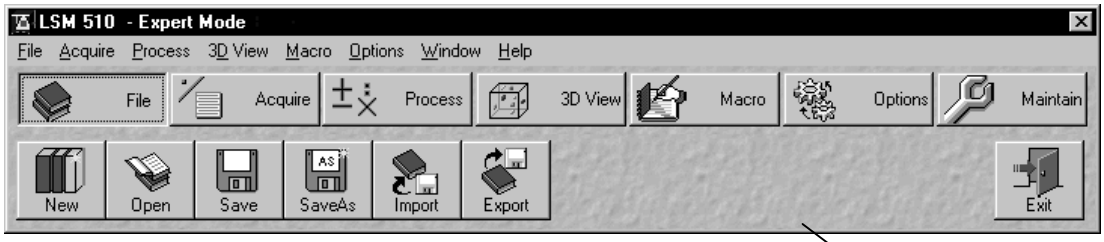


Fig. 4-149

- Click on the <Open> button.
  - This opens the "Open Database" window for selection of the database in which images have been stored.
- If you want to load a database in another folder (drive/directory), click on the arrow button to the right of the **Look in** box.
  - This opens a drop-down list box in which you can select from all available folders.

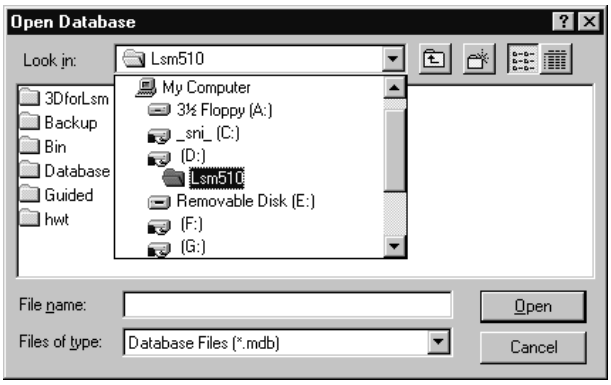


Fig. 4-150

The window displays the various Access image databases with the file extension ".mdb".

- Open the image database by a double click on the respective key icon (e.g. Multi Channel 2_0.mdb), or click on the name of the image database for selection and open it by clicking on the <Open> button.

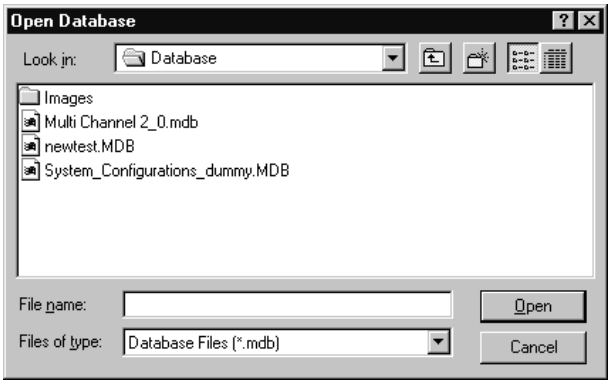


Fig. 4-151

- This opens a window, e.g. Multi Channel 2_0.mdb - LSM 510, with buttons which can be used to call up the individual images in the database and to have them presented in various ways.
- From the image database you can call up images in the following ways:


to the next image


back to the previous image


to the last image of the image database


to the first image of the image database

☞ The image number (Recordset) of the currently displayed image of an image series is indicated. You can browse through the series by dragging the slider using the mouse.

To view the slices in a stack one by one, drag the Slice slider.

Configuration data of a scan image

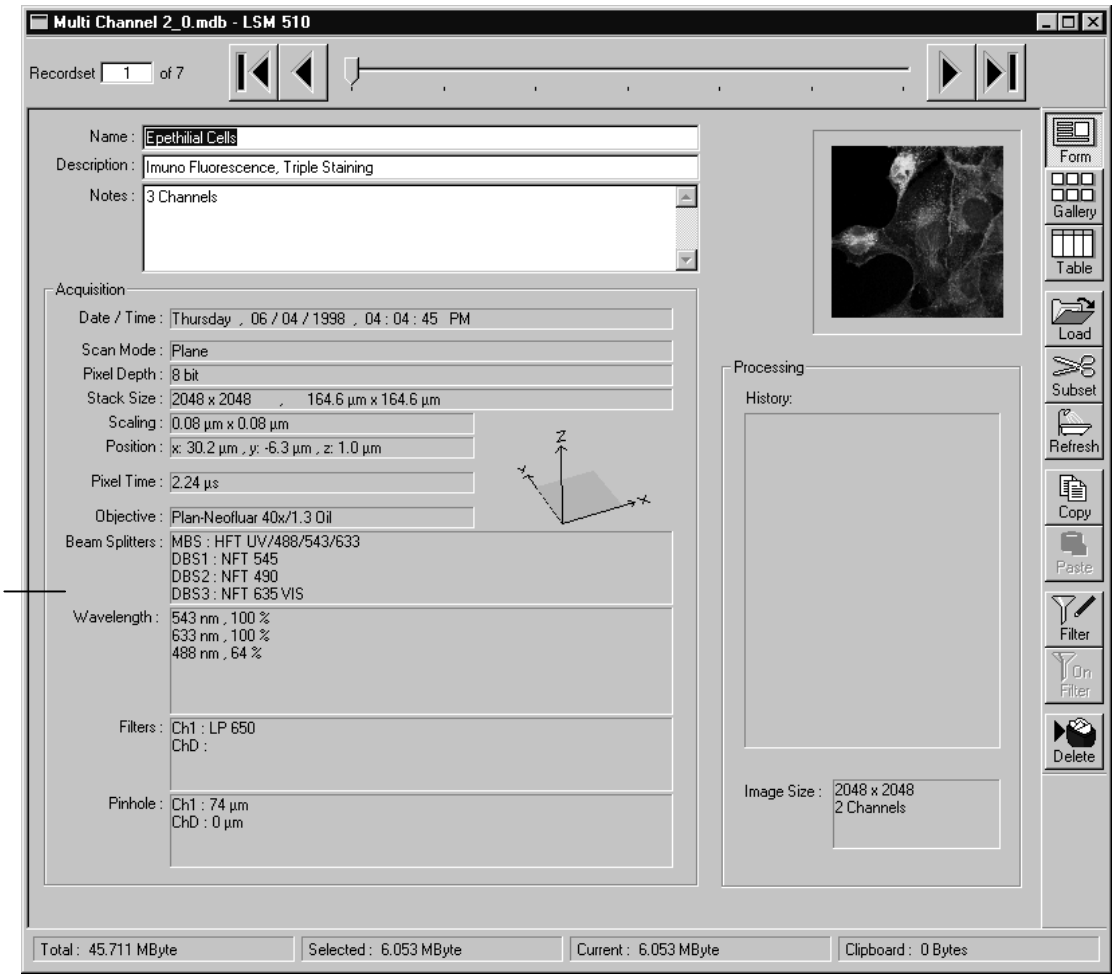

Multi Channel 2_0.mdb - LSM 510
Recordset 1 of 7
Name : Epethilial Cells
Description : Imuno Fluorescence, Triple Staining
Notes : 3 Channels
Acquisition
Date / Time : Thursday , 06 / 04 / 1998 , 04 : 04 : 45 PM
Scan Mode : Plane
Pixel Depth : 8 bit
Stack Size : 2048 x 2048 , 164.6 µm x 164.6 µm
Scaling : 0.08 µm x 0.08 µm
Position : x: 30.2 µm , y: -6.3 µm , z: 1.0 µm
Pixel Time : 2.24 µs
Objective : Plan-Neofluar 40x/1.3 Oil
Beam Splitters : MBS : HFT UV/488/543/633
DBS1 : NFT 545
DBS2 : NFT 490
DBS3 : NFT 635 VIS
Wavelength : 543 nm , 100 %
633 nm , 100 %
488 nm , 64 %
Filters : Ch1 : LP 650
ChD :
Pinhole : Ch1 : 74 µm
ChD : 0 µm
Processing
History:
Image Size : 2048 x 2048
2 Channels
Form
Gallery
Table
Load
Subset
Refresh
Copy
Paste
Filter
On Filter
Delete
Total : 45.711 MByte
Selected : 6.053 MByte
Current : 6.053 MByte
Clipboard : 0 Bytes

Fig. 4-152

- Click on the <Gallery> button.
  - All images of the image database, e.g. Multi Channel 2_0.mdb, (image series) are shown in a tiled arrangement on the screen.

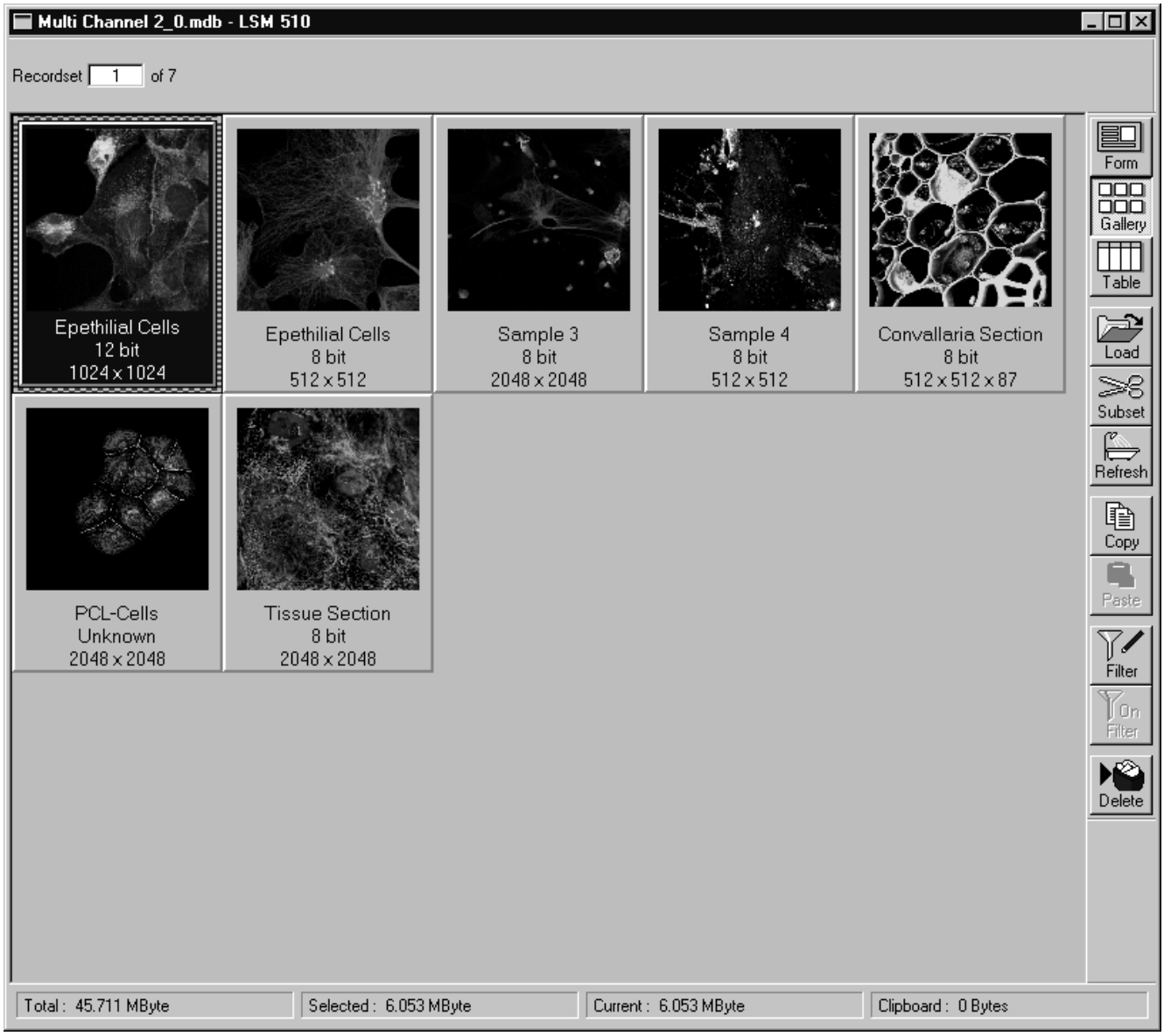


Fig. 4-153

- To select one of the images of the series for normal-size presentation, double-click on the desired image.

☞ The same can be achieved by clicking on the desired image in the gallery and then clicking on the <Load> button.

Image processing and analysis can be effected via the two-column toolbar (see section 4.8)

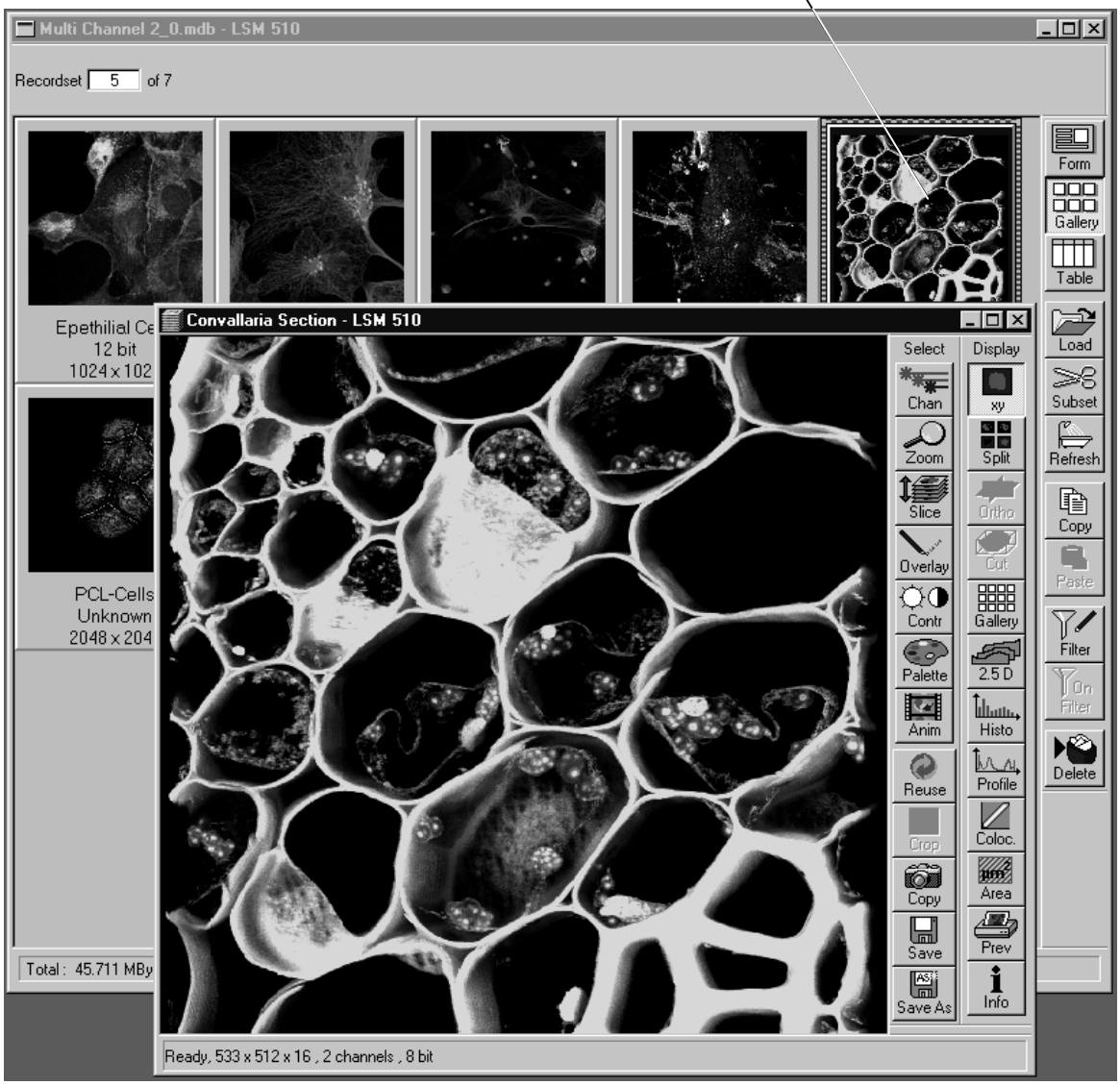


Fig. 4-154

4.9.3 Saving an image

- Click on the <File> button of the main menu toolbar.
  - This opens another, subordinate toolbar in the main menu.
- Click on the <Save> or <Save As> button.

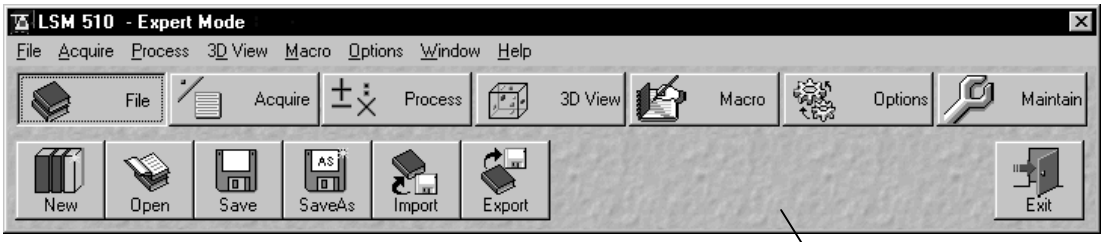


Fig. 4-155

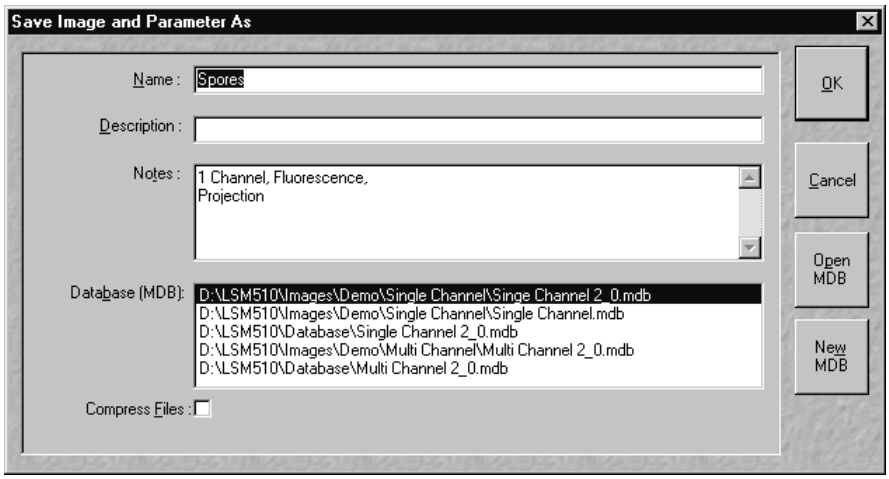


Fig. 4-156

Save Stores a newly created or changed image. Newly created images must be given a name and assigned to an existing or new database.

Save As Stores a previously stored and called up image under a different name.

Clicking on either of these buttons opens the "Save As" window to create and open an image database.

Click on the <Open MDB> button if you want to open an existing image database in which you want to save the current image. Click on the <New MDB> button if you want to create a new database to save the current image.

- Enter the name of the image in the **Name** textbox, e.g. Spores.
- Click on the <New MDB> button.
  - This opens the "Create New Database" window in which you can create a new image database.
- Enter the name of the database you want to create in the **File name** text box, e.g. "Single Channel 2_0".
- If you want to create the image database in a certain folder (drive/directory), click on the arrow button next to the **Create in** box.
  - This opens a drop-down list box showing all folders available for selection.
- After selection, click on the <Create> button.
  - This creates the image database in the selected drive and directory.

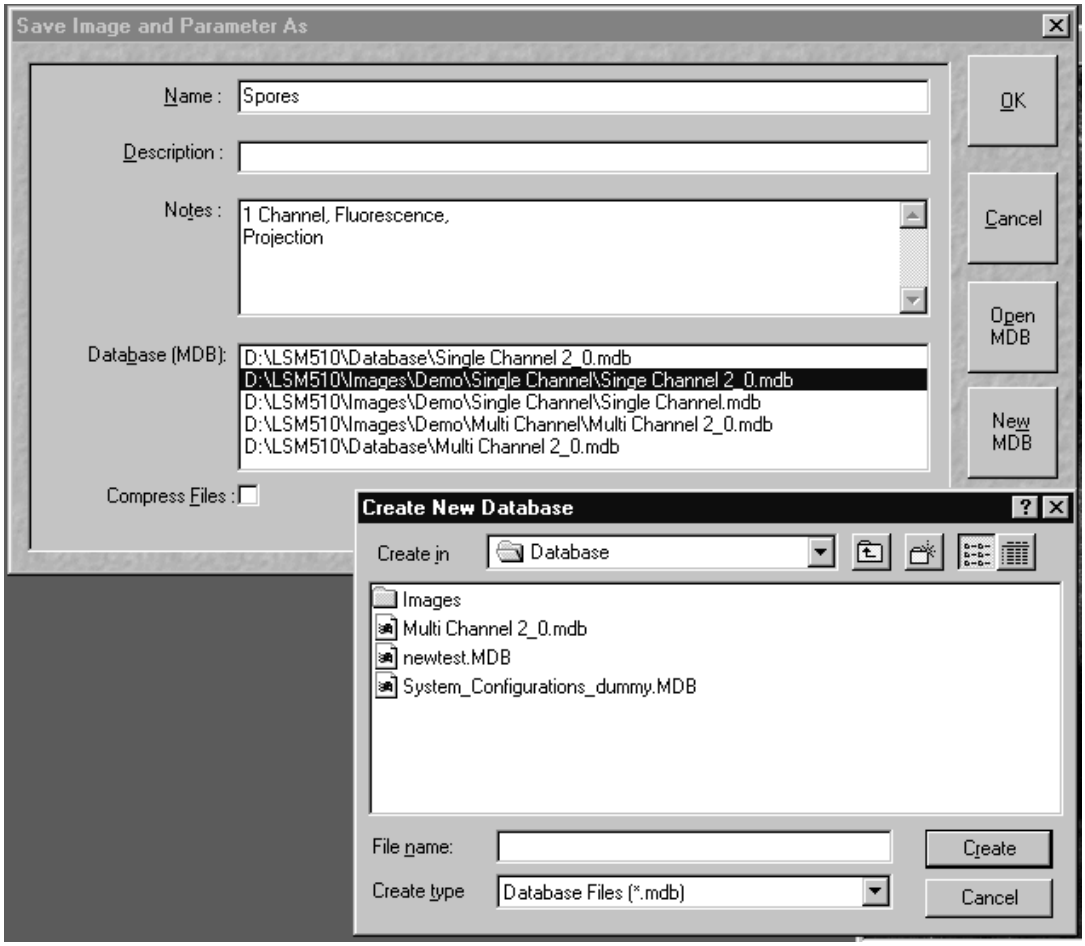


Fig. 4-157

- The "Single Channel 2_0.mdb-LSM510" window appears.
  - The window now shows the saved image.
  - The **Recordset** box indicates the current number of the image in the image series contained in this database.
- In the **Description** textbox you can enter, for example, the configuration of the image.
- In the **Notes** textbox you can enter further information about the image content.

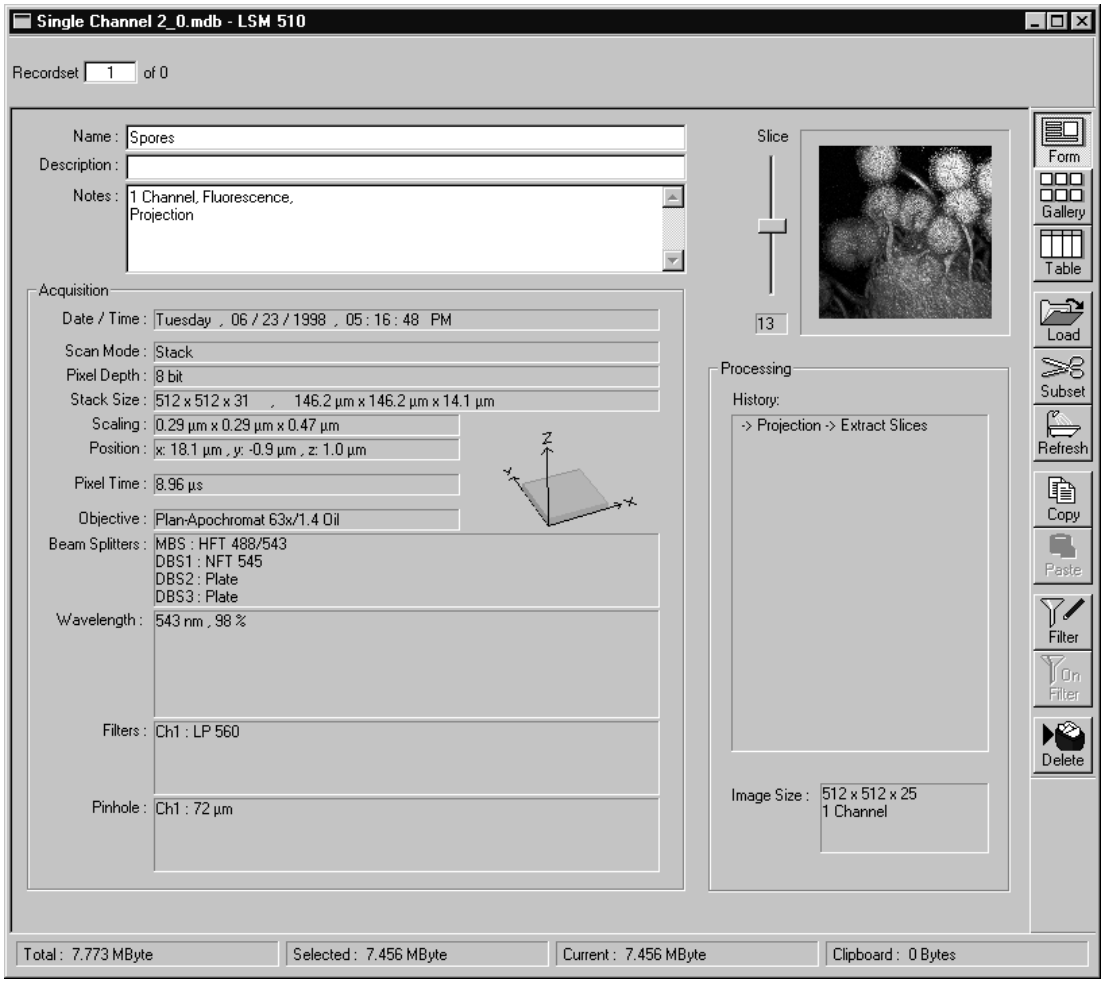

Single Channel 2_0.mdb - LSM 510
Recordset 1 of 0
Name : Spores
Description :
Notes : 1 Channel, Fluorescence, Projection
Slice
13
Acquisition
Date / Time : Tuesday , 06 / 23 / 1998 , 05 : 16 : 48 PM
Scan Mode : Stack
Pixel Depth : 8 bit
Stack Size : 512 x 512 x 31 , 146.2 µm x 146.2 µm x 14.1 µm
Scaling : 0.29 µm x 0.29 µm x 0.47 µm
Position : x: 18.1 µm , y: -0.9 µm , z: 1.0 µm
Pixel Time : 8.96 µs
Objective : Plan-Apochromat 63x/1.4 Oil
Beam Splitters : MBS : HFT 488/543
DBS1 : NFT 545
DBS2 : Plate
DBS3 : Plate
Wavelength : 543 nm , 98 %
Filters : Ch1 : LP 560
Pinhole : Ch1 : 72 µm
Processing
History:
-> Projection -> Extract Slices
Image Size : 512 x 512 x 25
1 Channel
Form
Gallery
Table
Load
Subset
Refresh
Copy
Paste
Filter
On Filter
Delete
Total : 7.773 MByte
Selected : 7.456 MByte
Current : 7.456 MByte
Clipboard : 0 Bytes

Fig. 4-158

☞ The "Acquisition" dialog box displays the parameters with which the scan image has been acquired.

4.9.4 Import of images

- Click on the <File> button of the main menu toolbar.
  - This opens another, subordinate toolbar in the main menu.

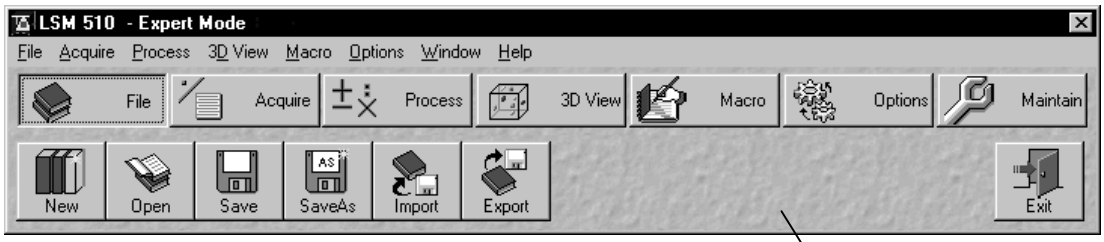


Fig. 4-159

Subordinate toolbar for the File menu item

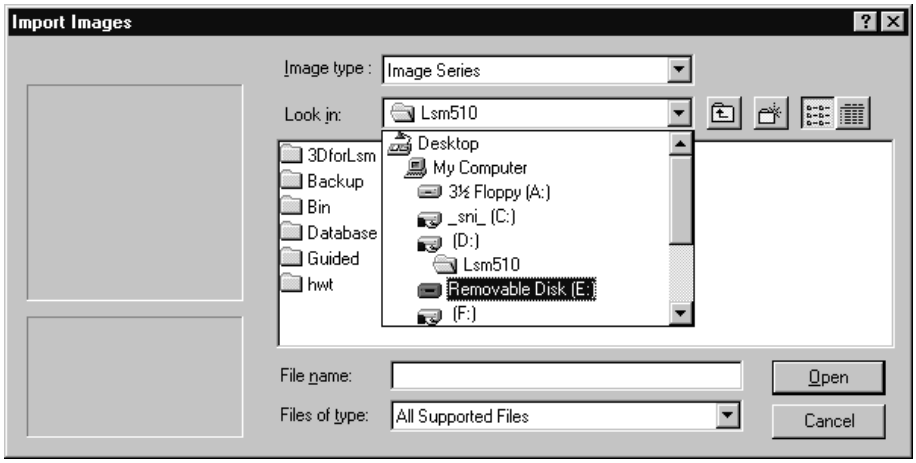


Fig. 4-160

- Click on the <Import> button.
  - This opens the **Import Image** dialog box.
- Select the data medium and the directory where the relevant image is contained.
- Select the image file by clicking on it and then click on <Open>.
  - The image is displayed in a new window

All the usual image formats (e.g. .tif, .jpg, .bmp, .pcx etc.) are supported.

4.9.5 Export of images

The <Export> function allows the export of both newly scanned images and images from the database. For this, the relevant image must be created or loaded.

- Click on the <File> button of the main menu toolbar.
  - This opens another, subordinate toolbar in the main menu.

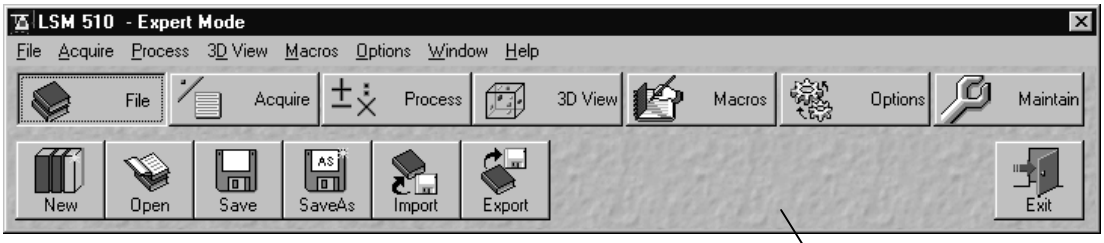


Fig. 4-161

Subordinate toolbar for the File menu item

- Load the image to be exported.
- Click on the <Export> button.
  - This opens the **Export Images and Data** dialog box.
- Under **Save in,** select the data medium and the directory to which the image is to be exported.
- Enter a name for the image under **File name**.
- Select the image format into which the image is to be exported under **Save as type**.
- Click on the <Save> button.
  - The image is stored on the relevant data medium / directory.

All the usual image formats (e.g. .tif, .jpg, .bmp, .pcx etc.) are supported.

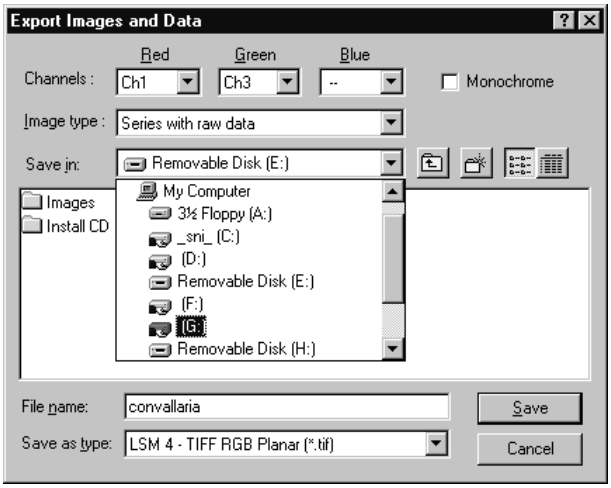


Fig. 4-162

☞ When stacks are exported, each frame is stored as an individual image.

Notes:

System backup

- A complete backup is contained on the enclosed optical disk.

User files backup

The following user-generated files need to be included in a backup procedure (keep directory structure):

- Image database files *.mdb (but not system_configuration_*.mdb
- LSM Image files *.lsm
- Exported images *.* (*.Tiff, *.LSM-Tiff, *.BMP, ...)
- Palette files lsm510 \ Palette \ *.lut
- Filter files lsm510 \ Filter \ *.krn
- Pinhole setting files lsm510 \ PH*.pos
- Log files lsm510 \ *.log

The following files generated during the system integration should also be included in a backup procedure:

- Parameter file for pinhole adjustment lsm510 \ *.set
- Parameter file after pinhole adjustment lsm510 \ *.adj
- Scanner files lsm510 \ bin \ *.bin
- Microscope stand database lsm510 \ database \ system_configuration_*.mdb

4.10 Macro

The macro function permits the recording, running and editing of command sequences and their allocation to buttons in the **Macro** main menu.

4.10.1 Macro language

"Visual Basic for Applications", called VBA in the following, is used as the Macro language. This language is well known through its widespread use as Macro language in the "Microsoft Word for Windows" and "Microsoft Excel for Windows" products. Experience with "Microsoft Visual Basic" would also be beneficial for macroprogramming of the LSM 510.

An Integrated Development Environment, called IDE in the following, is available for the editing and debugging of macros. IDE includes an "online help program" where the VBA language is described in detail.

Macros are stored in project files. One project file can include several macros.

4.10.2 Macro Control

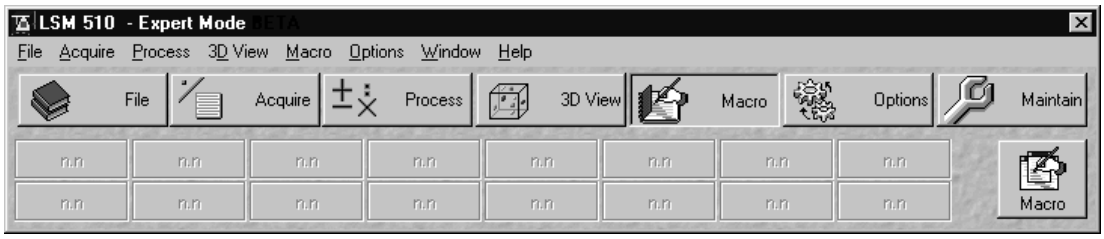


Fig. 4-163

- The <Macro / Macro> buttons in the main menu open the "Macro Control" window.

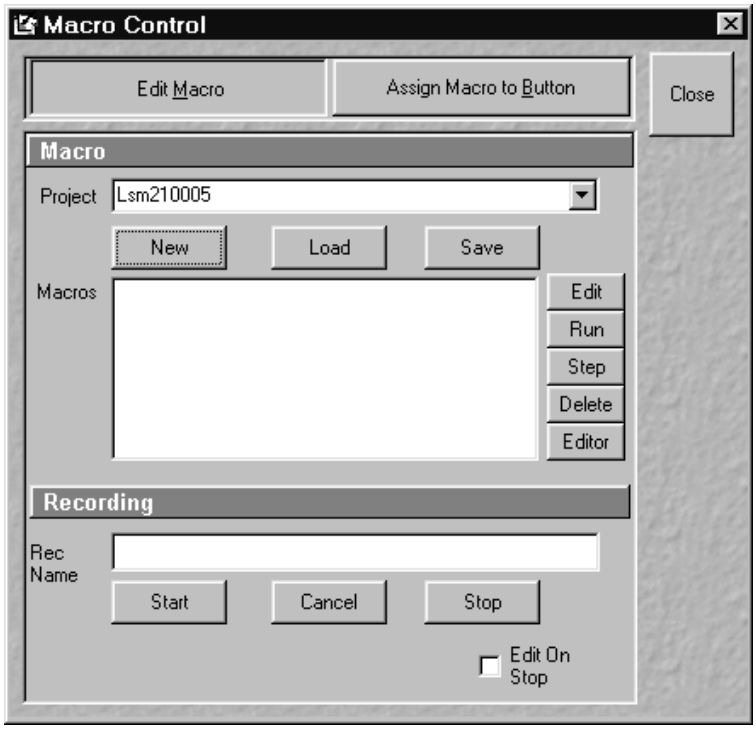


Fig. 4-164

This window allows you to manage project data and to allocate macros to the buttons in the main window. Before you can record or edit a macro, you have to create a project as follows:

- Press the <New> button to create a project name.

4.10.3 Recording and running of macros

- Before recording a command sequence, you can enter the name for the macro to be created in the **Rec Name** editing box of the "Recording" dialog box.

The following buttons are used for recording and running:

"Recording" dialog box

<Start> - starts recording.

<Stop> - stops recording.

"Macro" dialog box

<Run> - runs a macro.

Recorded macros are stored in main memory first. Before the macros can be assigned to the buttons in the **Macro** submenu, the project must be stored on the hard disk.

- Press the <Save> button under the project name in the "Macro Control" window and determine the file name in the file selection box.

4.10.4 Assignment of macros to the macro buttons in the *main window*

- Press the <Assign Macro to Button> button to switch to the "Define Buttons" dialog box.

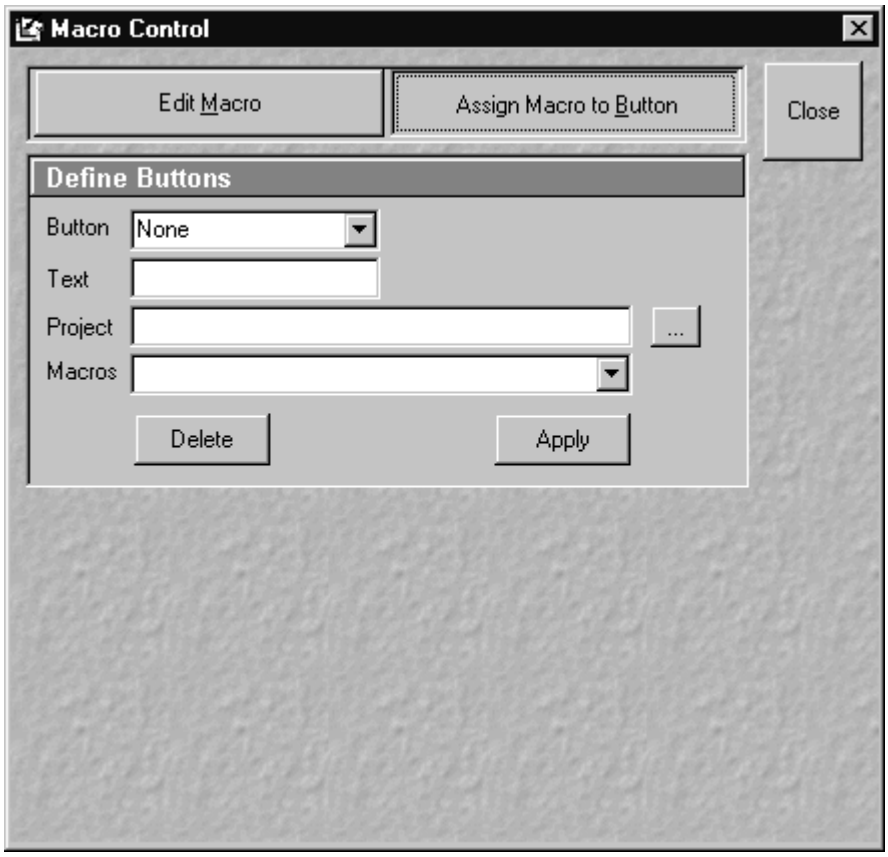


Fig. 4-165

- Select the button number from the "Button" box
- Select the button labelling in the "Text" editing box
- Select the name of the project file from the "Project" box using the < ... > button
- Select the macro name from the "Macros" box.
- Press the <Apply> button to assign the relevant macro to the specified button in the **Macro** toolbar.

4.10.5 Editing and debugging of macros

The <Edit> button activates IDE which allows macros to be edited and debugged. Under the "Help - Contents and Index" menu item, IDE contains detailed "online" help on its operation and on the VBA macro language. Therefore, only a few hints are provided in the following:

You should activate the required toolbars. We would recommend you to activate the "Debug" toolbar via the "View - Toolbars -Debug" menu item.

The following buttons in the toolbar can help you when debugging macros:


Starts running the command lines


Stops running the command lines


Interrupts processing of the command lines (pause)


Sets a breakpoint in the line with the text cursor.


Processes a command line and steps into subprocedures


Processes a command line and steps over subprocedures.


Exits the subprocedure (step out).


Displays the value of the marked expression (Watch). If nothing is marked, the value of the variable above the text cursor is displayed.


Activates the "Watch" window in which values of variables and expressions can be displayed. For this, text is marked in the code window and dragged into the "Watch" window. Variables can be modified in the "Watch" window.

In the left-hand edge of the code window you will find an arrow beside the current command line. A new current command line can be determined by moving the arrow via the mouse. This makes it possible to skip command lines or to process command lines several times.

4.11 Shut-Down Procedure

Never shut down the computer by its main switch while your LSM program is still active, or else you will lose the currently set operating parameters and the images just scanned.

In the "Settings for user" dialog window, which can be activated with the <Options/Settings> buttons, activate "Laser off on Exit" in the "Shutdown" register. The lasers will then automatically be switched off when you exit the LSM program.

4.11.1 Exiting the LSM program

- Close all open windows of the LSM program by clicking on the closing icon in the top right corner of each window.
  - This closes the respective window and removes the respective icons from the taskbar.
  - After all dialog windows have been closed, the "LSM 510 Switchboard" window appears.



Fig. 4-166

- Click on the <Exit> button.
  - This terminates the LSM program.
  - The monitor screen shows the desktop of the Windows NT operating system.

4.11.2 Running down the operating system

- Move the cursor to the bottom margin of the screen.
  - This opens the taskbar containing the <Start> button.
- Click on the <Start> button of the taskbar.
  - This opens a pop-up menu.
- Click on the "Shut Down" item.



Fig. 4-167

– This opens the "Shut Down Windows" window, in which you can select between **Shut down, Restart** and **Login**.

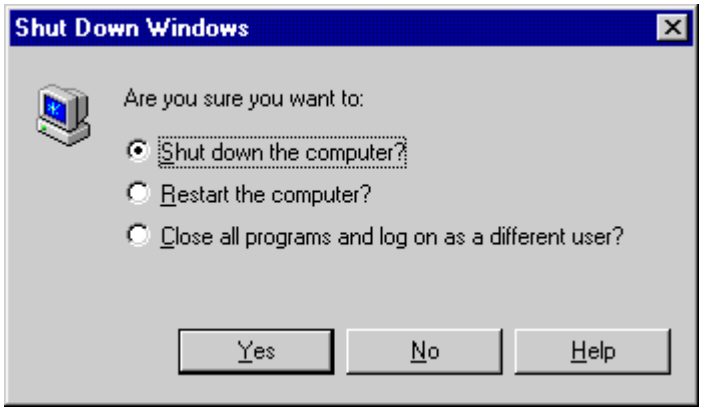


Fig. 4-168

- Unless already set by default, click on "Shut down the computer?".
- Click on the <Yes> button.

– The screen now displays the message

"Shutdown in Progress - Please wait while the system writes unsaved data to the disk."

– About 20 seconds after WINDOWS NT has been run down, the "Shutdown Computer" window appears which tells you that you can now turn off your computer.



Fig. 4-169

(3) Turning power off

- Throw the REMOTE CONTROL main switch to position "OFF".
  – This puts your LSM 510 microscope system, including the computer, off power.

4.12 Annex

4.12.1 Application-specific configurations

Application-specific configurations for module 510-1 (458/488, 543), 2 PMTs

	Lucifer Yellow	FITC (Cy2)	Rhod (Cy3, TexRed)
Laser	458	488	543
HFT	458	488	543
NFT 1	none	none	none
NFT 2			
NFT 3	none	none	none
EM 1	LP 475	LP 505	LP 560
EM 2			
EM 3			
EM 4			

	FITC (narrow band)	Rhodamine (narrow band)	FITC/Rhod
Laser	488	543	488, 543
HFT	488	543	488/543
NFT 1	none	none	NT 545
NFT 2			mirror
NFT 3	none	none	plate
EM 1	BP 505-550	BP 560-615	LP 560
EM 2			BP 505-530
EM 3			
EM 4			

Application-specific configurations for module 510-2 (488/568), 2 PMTs

	FITC (Cy2)	Rhod (Cy3, TexRed)	FITC/Rhod
Laser	488	568	488, 543
HFT	488	568	488/568
NFT 1	none	none	NT 570
NFT 2			mirror
NFT 3	none	none	plate
EM 1	LP 505	LP 585	LP 585
EM 2			BP 505-550
EM 3			
EM 4			

	FITC (narrow band)	Rhodamine (narrow band)
Laser	488	568
HFT	488	568
NFT 1	none	none
NFT 2		
NFT 3	none	none
EM 1	BP 505-550	BP 585-615
EM 2		
EM 3		
EM 4		

Application-specific configurations for module 510-3 (458/488, 543, 633), 3 PMTs

	Lucifer Yellow	FITC (Cy2)	Rhod (Cy3, TexRed)	Cy5
Laser	458	488	543	633
HFT	458	488	543	633
NFT 1	none	none	none	none
NFT 2				
NFT 3	none	none	none	none
EM 1	LP 475	LP 505	LP 560	LP 650
EM 2				
EM 3				
EM 4				

	FITC/Rhod	Rhod/Cy5	FITC/Cy5	FITC/Rhod/Cy5
Laser	488, 543	543, 633	488, 633	488, 543, 633
HFT	488/543	UV/488/543/633	UV/488/568/633	UV/488/543/633
NFT 1	NT 545	NT 635	NT 570	NT 635
NFT 2	mirror	plate	mirror	NT 545
NFT 3	plate	plate	plate	plate
EM 1	LP 560	LP 650	LP 650	LP 650
EM 2	BP 505-530		BP 505-550	BP 505-530
EM 3		BP 560-615		BP 560-615
EM 4				

	FITC (narrow band)	Rhodamine (nb)
Laser	488	543
HFT	488	543
NFT 1	none	mirror
NFT 2		plate
NFT 3	none	
EM 1	BP 505-550	
EM 2		
EM 3		BP 560-615
EM 4		

Application-specific configurations for module 510-4 (488/568, 633), 3 PMTs

	FITC (Cy2)	Rhod (Cy3, TexRed)	Cy5
Laser	488	568	633
HFT	488	568	633
NFT 1	none	none	none
NFT 2			
NFT 3	none	none	none
EM 1	LP 505	LP 585	LP 650
EM 2			
EM 3			
EM 4			

	FITC/Rhod	FITC/Cy5	Rhod/Cy5	FITC/Rhod/Cy5
Laser	488, 568	488, 633	568, 633	488, 568, 633
HFT	488/568	UV/488/568/633	UV/488/568/633	UV/488/568/633
NFT 1	NT 570	NT 570	NT 635	NT 635
NFT 2	mirror	mirror	plate	NT 570
NFT 3	plate	plate	plate	plate
EM 1	LP 585	LP 650	LP 650	LP 650
EM 2	BP 505-550	BP 505-550		BP 505-550
EM 3			BP 585-615	BP 585-615
EM 4				

	FITC (narrow band)	Rhodamine (nb)
Laser	488	568
HFT	488	568
NFT 1	none	none
NFT 2		
NFT 3	none	none
EM 1	BP 505-550	BP 585-615
EM 2		
EM 3		
EM 4		

Application-specific configurations for module 510-5 (351/364, 458/488, 543), 3 PMTs

	DAPI	Lucifer Yellow	FITC (Cy2)	Rhod (Cy3, TexRed)
Laser	364	458	488	543
HFT	UV (375)	458	488	543
NFT 1	none	none	none	none
NFT 2				
NFT 3	none	none	none	none
EM 1	LP 385	LP 475	LP 505	LP 560
EM 2				
EM 3				
EM 4				

	DAPI/FITC	DAPI/Rhod	FITC/Rhod	DAPI/FITC/Rhod
Laser	364, 488	364, 543	488, 543	364, 488, 543
HFT	UV/488	UV/543	488/543	UV/488/543/633
NFT 1	NT 490	NT 490	NT 545	NT 545
NFT 2	mirror	mirror	plate	NT 490
NFT 3	plate	plate	plate	plate
EM 1	LP 505	LP 560	LP 560	LP 560
EM 2	BP 385-470	BP 385-470		BP 385-470
EM 3			BP 505-530	BP 505-530
EM 4				

	DAPI (nb)	FITC (nb)	Rhodamine (nb)
Laser	364	488	543
HFT	UV (375)	488	543
NFT 1	mirror	mirror	none
NFT 2	mirror	plate	
NFT 3			none
EM 1			BP 560-615
EM 2	BP 385-470		
EM 3		BP 505-550	
EM 4			

Application-specific configurations for module 510-6 (351/364, 458/488, 543, 633), 4 PMTs

	DAPI	Lucifer Yellow	FITC (Cy2)	Rhod (Cy3, TexRed)	Cy5
Laser	364	458	488	543	633
HFT	UV (375)	458	488	543	UV/488/543/633
NFT 1	none	none	none	none	none
NFT 2					
NFT 3	none	none	none	none	none
EM 1	LP 385	LP 475	LP 505	LP 560	LP 650
EM 2					
EM 3					
EM 4					

	DAPI/FITC	DAPI/Rhod	DAPI/Cy5	FITC/Rhod	FITC/Cy5
Laser	364, 488	364, 543	364, 633	488, 543	488, 633
HFT	UV/488	UV/543	UV/488/543/633	488/543	UV/488/543/633
NFT 1	mirror	NT 545	NT 545	NT 545	NT 570
NFT 2	NT 490	mirror	mirror	plate	plate
NFT 3		plate	plate	plate	plate
EM 1		LP 560	LP 505	LP 560	LP 650
EM 2	BP 385-470	BP 385-470	BP 385-470		
EM 3	LP 505			BP 505-530	BP 505-550
EM 4					

	Rhod/Cy5	DAPI//FITC/Rhor	DAPI/FITC/Cy5	DAPI/Rhod/Cy5	FITC/Rhod/Cy5
Laser	543, 633	364, 488, 543	364, 488, 633	364, 543, 633	488, 543, 633
HFT	UV/488/543/633	UV/488/543/633	UV/488/543/633	UV/488/543/633	UV/488/543/633
NFT 1	plate	NT 545	NT 570	NT 545	NT 545
NFT 2		NT 490	NT 490	mirror	plate
NFT 3	NT 635 Vis	plate	plate	NT 635 Vis	NT 635 Vis
EM 1	LP 650	LP 560	LP 650	LP 650	LP 650
EM 2		BP 385-470	BP 385-470	BP 385-470	
EM 3		BP 505-530	BP 505-550		BP 505-530
EM 4	BP 560-615			BP 560-615	BP 560-615

	DAPI/FITC/Rhod/ Cy5	DAPI (nb)	FITC (nb)	Rhodamine (nb)
Laser	364, 488, 543, 633	364	488	543
HFT	UV/488/543/633	UV (375)	488	543
NFT 1	NT 545	mirror	mirror	mirror
NFT 2	NT 490	mirror	plate	plate
NFT 3	NT 635 Vis			
EM 1	LP 650			
EM 2	BP 385-470	BP 385-470		
EM 3	BP 505-530		BP 505-550	BP 560-615
EM 4	BP 560-615			

Application-specific configurations for module 510-7 (351/364, 488/568, 633), 4 PMTs

	DAPI	FITC (Cy2)	Rhod (Cy3, TexRed)	CY5	DAPI/FITC
Laser	364	488	568	633	364, 488
HFT	UV (375)	488	568	UV/488/568/633	UV/488
NFT 1	none	none	none	none	NT 490
NFT 2					mirror
NFT 3	none	none	none	none	plate
EM 1	LP 385	LP 505	LP 585	LP 650	LP 505
EM 2					BP 385-470
EM 3					
EM 4					

	DAPI/Rhod	DAPI/Cy5	FITC/Rhod	FITC/CY5	Rhod/Cy5
Laser	364, 568	364, 633	488, 568	488, 633	568, 633
HFT	UV/568	UV/488/568/633	488/568	UV/488/568/633	UV/488/568/633
NFT 1	NT 490	NFT 570	NT 570	NT 570	plate
NFT 2	mirror	mirror	plate	plate	
NFT 3	plate	plate	plate	plate	NT 635 Vis
EM 1	LP 585	LP 650	LP 585	LP 650	LP 650
EM 2	BP 385-470	BP 385-470			
EM 3			BP 505-550	BP 505-550	
EM 4					BP 585-615

	DAPI/FITC/Rhod	DAPI/FITC/Rhod/ Cy5	DAPI/FITC/Cy5	DAPI/Rhod/Cy5	FITC/Rhod/Cy5
Laser	364, 488, 568	364, 488, 568, 633	364, 488, 633	364, 568, 633	488, 568, 633
HFT	UV/488/568/633	UV/488/568/633	UV/488/568/633	UV/488/568/633	UV/488/568/633
NFT 1	NT 570	NT 570	NT 570	NT 570	NT 570
NFT 2	NT 490	NT 490	NT 490	mirror	plate
NFT 3	plate	NT 635 Vis	plate	NT 635 Vis	NT 635 Vis
EM 1	LP 585	LP 650	LP 650	LP 650	LP 650
EM 2	BP 385-470	BP 385-470	BP 385-470	BP 385-470	
EM 3	BP 505-550	BP 505-550	BP 505-550		BP 505-550
EM 4		BP 585-615		BP 585-615	BP 585-615

	DAPI (nb)	FITC (nb)	Rhodamine (nb)
Laser	364	488	568
HFT	UV (375)	488	568
NFT 1	mirror	none	none
NFT 2	mirror		
NFT 3		none	none
EM 1		BP 505-550	BP 585-615
EM 2	BP 385-470		
EM 3			
EM 4			

Application-specific configurations for module 510-8 (458/488, 543, 633), 2 PMTs

	Lucifer Yellow	FITC (Cy2)	Rhod (Cy3)	Cy5
Laser	458	488	543	633
HFT	458	488	543	633
NFT 1	none	none	none	none
NFT 2				
NFT 3	none	none	none	none
EM 1	LP 475	LP 505	LP 560	LP 650
EM 2				
EM 3				
EM 4				

	FITC/Rhod	FITC/Cy5	Rhod/Cy5
Laser	488, 543	488, 633	543, 633
HFT	488/543	UV/488/543/633	UV/488/543/633
NFT 1	NT 545	NT 570	NT 635 Vis
NFT 2	mirror	mirror	mirror
NFT 3	plate	plate	plate
EM 1	LP 560	LP 650	LP 650
EM 2	BP 505-530	BP 505-550	BP 560-615
EM 3			
EM 4			

	FITC (nb)	Rhod (nb)
Laser	488	543
HFT	488	543
NFT 1	none	none
NFT 2		
NFT 3	none	none
EM 1	BP 505-550	BP 560-615
EM 2		
EM 3		
EM 4		

Application-specific configurations for module 510-9 (351/364, 458/488, 543), 2 PMTs

	DAPI	Lucifer Yellow	FITC (Cy2)	Rhod (Cy3)
Laser	351/364	458	488	543
HFT	UV (375)	458	488	543
NFT 1	none	none	none	none
NFT 2				
NFT 3	none	none	none	none
EM 1	LP 385	LP 475	LP 505	LP 560
EM 2				
EM 3				
EM 4				

	DAPI/FITC	DAPI/Rhod	FITC/Rhod
Laser	364, 488	364, 543	488, 543
HFT	UV/488	UV/543	488/543
NFT 1	NT 490	NT 545	NT 545
NFT 2	mirror	mirror	mirror
NFT 3	plate	plate	plate
EM 1	LP 505	LP 560	LP 560
EM 2	BP 385-470	BP 385-470	BP 505-530
EM 3			
EM 4			

	DAPI (nb)	FITC (nb)	Rhod (nb)
Laser	351/364	488	543
HFT	UV (375)	488	543
NFT 1	mirror	none	none
NFT 2	mirror		
NFT 3		none	none
EM 1		BP 505-550	BP 560-615
EM 2	BP 385-470		
EM 3			
EM 4			

4.12.2 Filter change in the detection beam path of channels 1 and 2

For optimum investigation of specimens it is useful to employ filter wheels permitting motor-controlled change between different filters for narrow-band or broad-band detection depending on the wavelength. The number of filters is limited by the capacity of the filter wheel. The change of the filter wheel as a whole involves complete readjustment.

The filter wheels of channels 1 and 2 of the Scanning Module have a change position in which a filter, including its mount, can be changed in a reproducible position without requiring readjustment. The filters can be rotated in their cells, and with the light path being eccentric relative to the filter center, the best transmission area of the filter for the respective wavelength or pass range can be found by rotating the filter. This is very important for the investigation of specimens of low emission.

Filter change

- By software control, move filter wheel (4-1/5) to the change position.
- Pull cover cap (4-1/1) off the Scanning Module.
- Use the wire stirrup (4-1/2) to pull the filter mount (4-1/4) with the filter (4-1/3) out of the guide well.
- Change filter to suit the application.

☞ The filter is rotatable in its mount, allowing adjustment for finding the best transmission area of the filter for the wavelength used.

- Enter the designation of this particular filter into the System Software database.

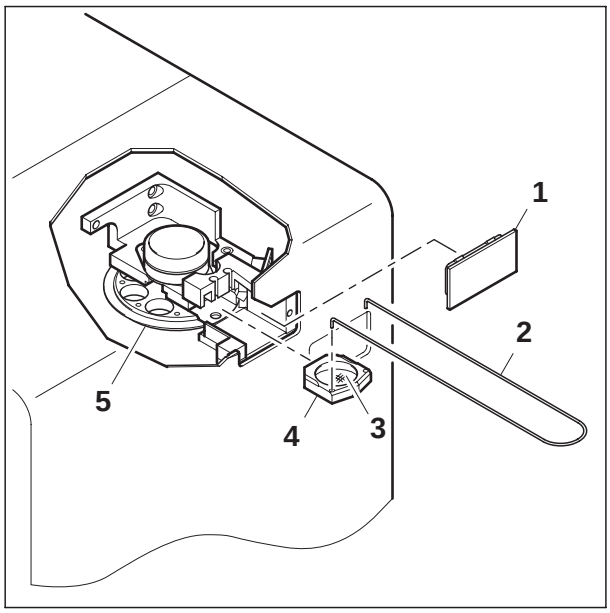


Fig. 4-170 Filter change

4.12.3 Detaching / Attaching the Scanning Module from / to Microscope Stands

Tool needed: 3 mm Allen key

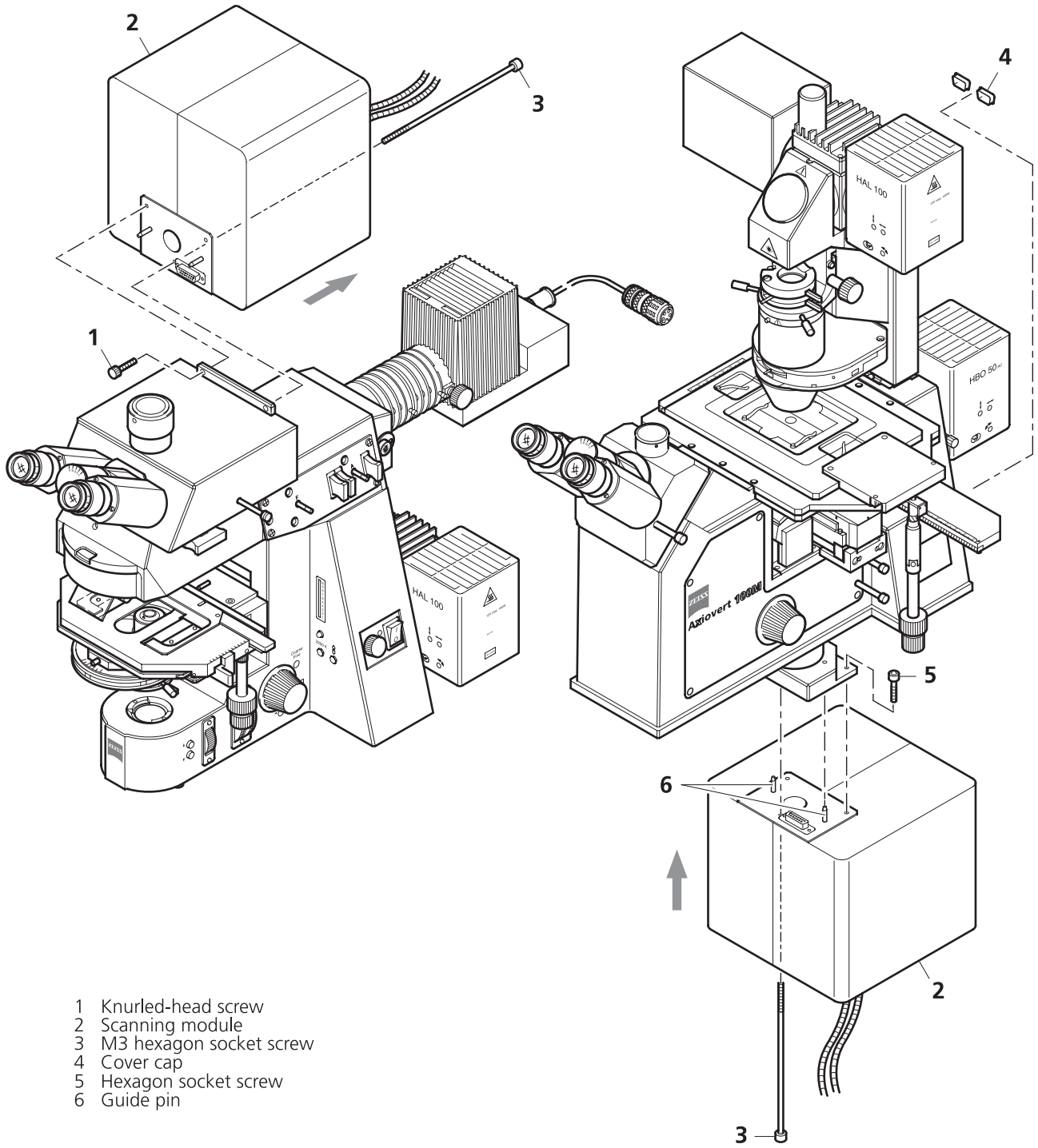
☞ The user can remove the Scanning Module from one microscope and attach it to another within a few minutes. **No adjustment** is required after the change-over. Described below is the change-over from an Axioplan to an Axiovert 100 M in baseport configuration.

⚠ **Before** the change-over, **shut down** the system as described in section 4.12 in order to avoid damage to the system and loss of data.

- Turn out both knurled-head screws (4-1/1) at the Scanning Module (4-1/2) fitted to the Axioplan.
- Turn out M3 hexagon socket screw (4-1/3) with the Allen key.
- Cautiously pull Scanning Module off the Axioplan stand.
- Attach Scanning Module to the baseport of the Axiovert, minding the guide pins (4-1/6), and secure it with the M3 hexagon socket screw (4-1/3).
- Fasten Scanning Module to the baseport with two hexagon socket screws (4-1/5), using an offset Allen key.

☞ As the Scanning Module is heavy, weighing about 14 kg, it is easier if the changeover is carried out by two persons.

- Pull off covering caps (4-1/4) from the CAN-BUS and RS232 interface ports at the rear of the Axiovert, remove the two cables 457411-9011 (CAN-BUS) and 457411-9012 (RS 232) from the Axioplan, plug them into the Axiovert and secure them there.
- Switch the LSM 510 on with the REMOTE switch.
- Click on the "Axiovert.ico" icon to update the system database with the new database of the Axiovert 100 M microscope.
- Restart the LSM program



1 Knurled-head screw
2 Scanning module
3 M3 hexagon socket screw
4 Cover cap
5 Hexagon socket screw
6 Guide pin

Fig. 4-171 Change-over of the Scanning Module

4.12.4 Hints on the use of the HRZ 200 fine focusing stage

4.12.4.1 General description

The HRZ 200 fine focusing stage is a compact attachment for the Axioplan 2 MOT and Axiovert 100 M microscope stages, which allows the particularly fast and high-precision fine focusing of the object. The HRZ 200 permits fine focusing over a range of 200 µm, with the smallest step width being less than 10 nm, reproducibility better than 40 nm, and the maximum speed amountig to 10 Hz. The stage allows the use of specimens with a weight of less than 100 g.

The HRZ 200 is not used if manual coarse focusing is performed. To position the objective in relation to the optical z-axis, the standard xy-microscope stage is used.

The HRZ 200 features a mount for standard object carriers of 76 mm x 26 mm x 1 mm and a milled-out receptacle for ∅ 36 mm x 1 mm Petri dishes.

4.12.4.2 Application fields

- High-precision fine focusing and translation of the object along the optical axis.
- Fast and high-precision mounting of one-dimensional *z*-line sections.
- Fast and high-precision mounting of two-dimensional *r*-*z*-longitudinal sections.
- Fast and high-precision mounting of *xy*-*z*-stacks for the three-dimensional reconstruction of the object.
- Exact measurement of Point-Spread-Functions for deconvolution.

The HRZ 200 can only be used in the Expert Mode.

4.12.4.3 Operation

Fine focusing

- Select the <Acquire> button from the LSM 510 main menu.
- Select the <Stage> button. The following menu appears:

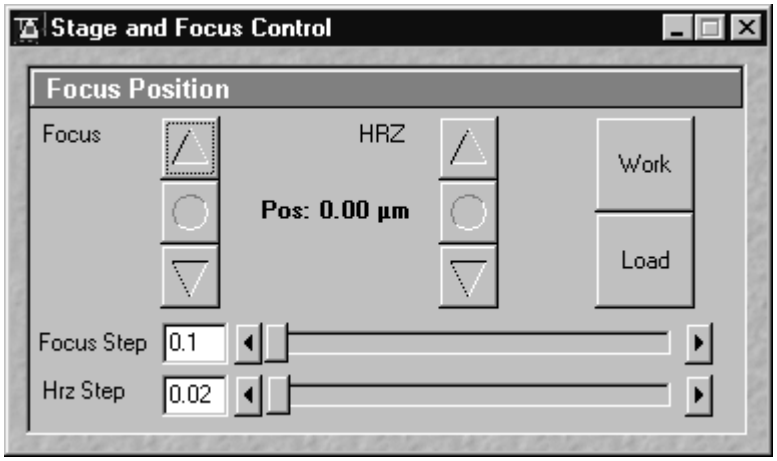


Fig. 4-172

- The <Hrz Step> slider is used to set the step width of the fine focusing stage.
- Use the arrows of the <HRZ> slider to move the fine focusing stage upwards or downwards in steps. Press the <O> button to reset the display to the value 0 and to move the fine-focusing stage to the zero position.
- The motor focus of the stand is operated in the same way via the relevant buttons. Moving into the <Work> or <Load> position is always performed via the motor focus and not via the HRZ.

Z Mode via the HRZ 200

This mode is used to acquire two- or three-dimensional stacks of images in different z-positions, depending on whether <Line> or <Frame> scan has been activated. The benefit of this mode over <Z Sectioning> via the motor focus is that the z-resolution can be increased by up to 20 times using the HRZ 200 and that the image information can be obtained up to 100 times faster with the HRZ 200.

- Select the <Z Mode> button in the "Scan Control" dialog. The <Z Settings> window will open.
- Select the <Hyperfine Z Sectioning> button. The following menu appears:

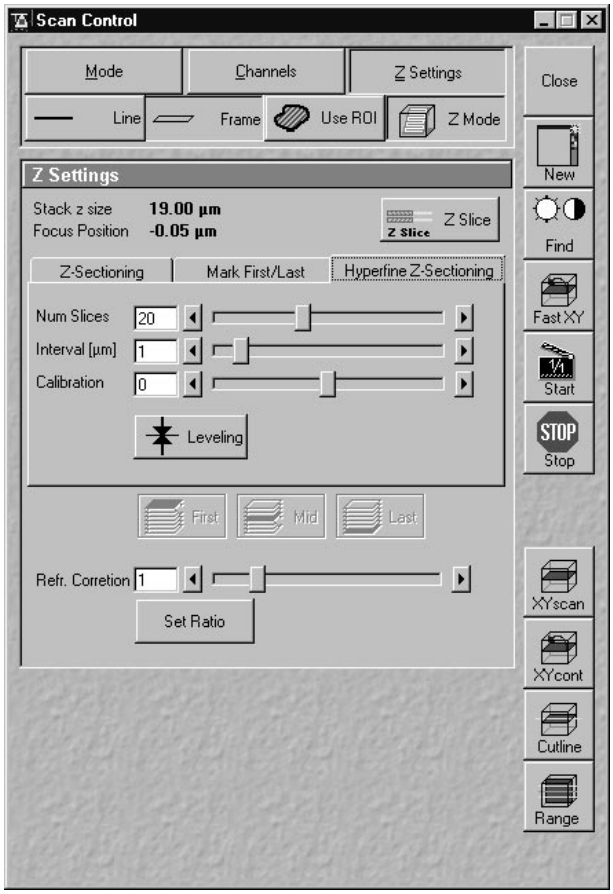


Fig. 4-173

- Optimization of system settings and the acquisition of the image stack is now performed in the same way as described in section 4.5.4.2 for the *Z Mode* with motor focus. The function of all controls is identical. The setting of <Current Slice> is made automatically.

- Clicking on the additional <Leveling> button moves the HRZ 200 to the zero position, while the motor focus moves into the opposite direction at the same time, i.e. the position of the object in relation to the objective remains unchanged. This function is used to set defined initial conditions.
- The <Calibration> slider must normally be left in the default position 0. Calibration is required only if the examined image field is located clearly outside the center of the specimen carrier on the HRZ 200. Calibration is described in the next section.

4.12.4.4 Additional information on the operation

The HRZ 200 fine-focusing stage is a high-precision, sensitive accessory for the LSM 510 from Carl Zeiss and must therefore be treated carefully. High mechanical stress, such as the use of specimens weighing more than 100 g or the application of pressure or knocks on the movable stage tongue, can result in damage and therefore in failure of the stage function.

To be able to fully utilize the outstanding precision attainable with the fine focusing stage, anything which could interfere with its operation, especially mechanical knocks and impact of the LSM components, should be avoided. We would recommend you to always use the actively vibration-damped Kinetics stage (available as accessory under the order number 1007 508 or 1007 512) as the base for the setup of LSM systems containing the HRZ 200 stage.

The specifications of the stage are obtained only after a heating phase of approx. 30 minutes. Furthermore, the installation conditions for the LSM system must be observed.

The maximum reproducibility (better than 40 nm) for moving to an absolute position in z is achieved by always moving to the required position from below.

Fine focusing is performed mechanically via an inclined position of the stage tongue. Therefore, the lifting range z at the location of the image field depends on the position of the HRZ in relation to the optical axis. This means: if the user shifts the object on the microscope stage to the right via the HRZ 200, the lift will be different from the one in the zero position of the stage (max. 200 µm) and also from the one after a shift of the stage to the left.

If the LSM system is equipped with a motorized scanning stage, this shift is read back to Δx and the lift is calibrated automatically if the zero position of the HRZ has been matched to the zero position of the scanning stage via an initialization run. For this, activate the <Stage> dialog in the <Acquire> menu. Then position the scanning stage in such a way that the optical axis of the microscope corresponds to the zero position of the HRZ, i.e. to the center of the specimen holder in the stage tongue. Then perform initialization by pressing the <HRZ Null> button. This step must be repeated after every new start of the system. Also see the notes on the operation of the motorized scanning stages.

If the system is equipped with a manual microscope stage, the user has the option of performing the calibration by entering the Δx shift in mm via the <Calibration> slider.

The shift is read off from the microscope stages. In the case of the manual Axioplan 2 stage, Δx can be read directly from the scale adhered to the front of the stage. In the case of the manual Axiovert 100 stage, a scale is located on the right of the knob, where the 45 mm Δx shift relative to the zero position of the microscope stage can be read off. The Δx value is positive for both stages if shift from the zero position is made to the right and negative if the shift is made to the left.

On account of the inclined position of the stage tongue, the object is also shifted laterally during the fine focusing motion. This lateral shift is negligibly small if, as recommended by us, specimen carriers with thickness 1.0 mm are used exclusively. Otherwise, the marked lateral shift of the object during fine focusing can result in image distortion. For the same reason, Petri dishes without fixation ring must be used exclusively.

The nosepiece of the Axiovert stand is moved to the load position prior to switching off the LSM system and the HRZ 200 is then moved to the lowest position to avoid damage of the objective or object by a possible collision. The user must refocus after start-up of the system. Before an objective change in the Axiovert or the Axioplan, the nosepiece and the microscope stage must be moved to the Load Position by the user, and then back to the Work Position to prevent the objectives from hitting the HRZ components. This is performed automatically if the objectives are changed menu-controlled via the relevant buttons of the LSM program.

The HRZ 200 for the Axiovert 100 M (1013 186) or for the Axioplan 2 MOT (1013 187) can be attached to the following standard microscope stages:

- mechanical stage 85 x 130 for Axiovert (45 13 39)
- scanning stage DC 100 x 90 for Axiovert (45 17 40)
- mechanical stages 75 x 50 for Axioplan (45 35 05, 45 35 02-99 04, 45 35 07)
- scanning stage DC 4“ x 4“ for Axioplan (45 35 85-99 01)

In the case of the last configuration, the object plane is shifted upwards so that Köhler illumination and classical transmitted-light microscopy will no longer be possible because the condenser cannot be moved sufficiently close to the object.

The user will not have to deal with any other restrictions.

4.12.5 Scanning stages

The following software description applies to systems which are equipped with a scanning stage.

4.12.5.1 Routine Mode

In the Routine Mode, the **Standard Examination Methods** panel enables you to reach the "Routine Mode - Microscope Setup" window

- Clicking on the <Stage> button opens the following window.

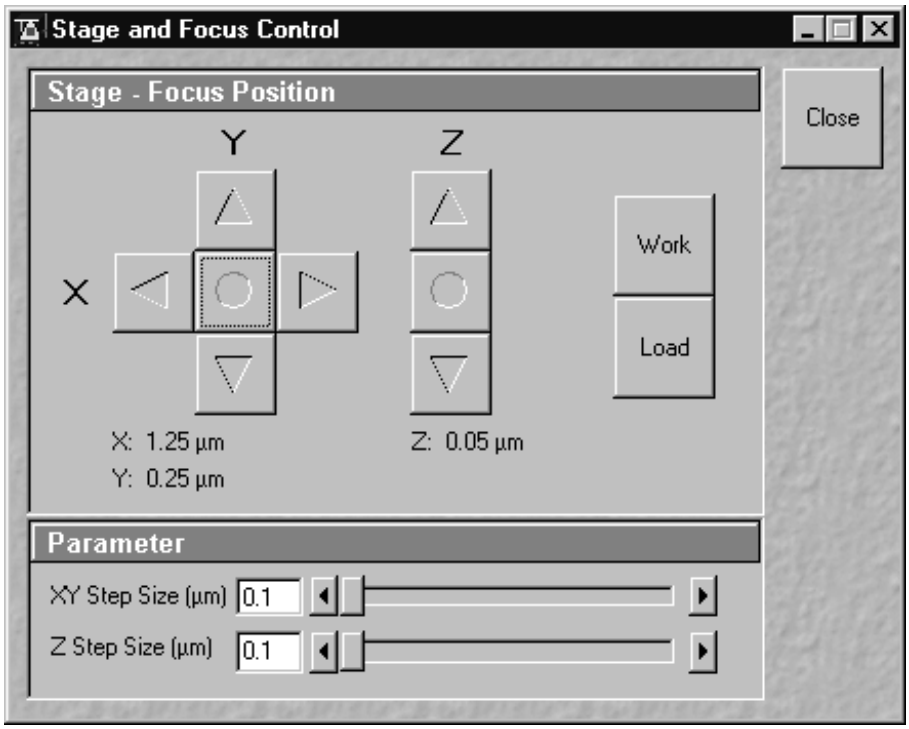


Fig. 4-174

This menu enables you to activate both the scanning stage and the motor focus.

The **Stage - Focus Position** panel includes the function keys for the performance of defined moves and the display of the current X, Y and Z positions.

The lower **Parameter** panel allows setting of the step width for X/Y and Z.

XY Step Size

The required step size for the scanning stage can be set in three ways:

1.) by shifting the slider

2.) by clicking on the arrow keys;

- clicking on the right arrow key increases the step width,
- clicking on the left arrow key decreases the step width.
- One click increases/decreases the step width by 1 μm.
- Pressing the **CTRL** key changes the step width by 0.1 μm.
- Pressing the **SHIFT** key changes the step width by 10 μm.

3.) by overwriting the displayed value

Values between 1 μm and 1000 μm can be entered for <XY Step Size>.

Z Step Size

The size of the focus step is set in the same way as the stage steps.

0.1 μm is the smallest value which can be set and 100 μm the highest.

- Clicking on the arrow keys changes the step size by 1 μm.
- Pressing the CTRL key and clicking changes the step size by 0.05 μm.
- Pressing the Shift key and clicking changes the step size by 10 μm.

Stage - Focus Position

<X> Moves and <Y> Moves

- Clicking on the <and> arrow keys triggers individual X or Y moves of the size set before in the parameter window.
- Pressing on O moves the stage to the zero position and resets the display to 0.

<Z> Moves

- Clicking on the **Up** arrow key moves the specimen stage/nosepiece upwards (the distance between objective and specimen is reduced).
- Clicking on the Down arrow keys moves the specimen stage/nosepiece downwards (the distance between objective and specimen is increased).

<Load>

- Clicking on the <Load> button lowers the specimen stage/nosepiece to make it easier for you to change the specimen (or objective).

<Work>

- Pressing the <Work> button moves the specimen stage/nosepiece back to the Work position. This is the position last set before the <Load> button was pressed.

The Routine Mode does not feature a **Mark & Find** function.

Of course, you can also use the joystick control panel to control the scanning stage.

4.12.5.2 Expert Mode

The following window opens in the Expert Mode after clicking on the Stage button in <Acquire>:

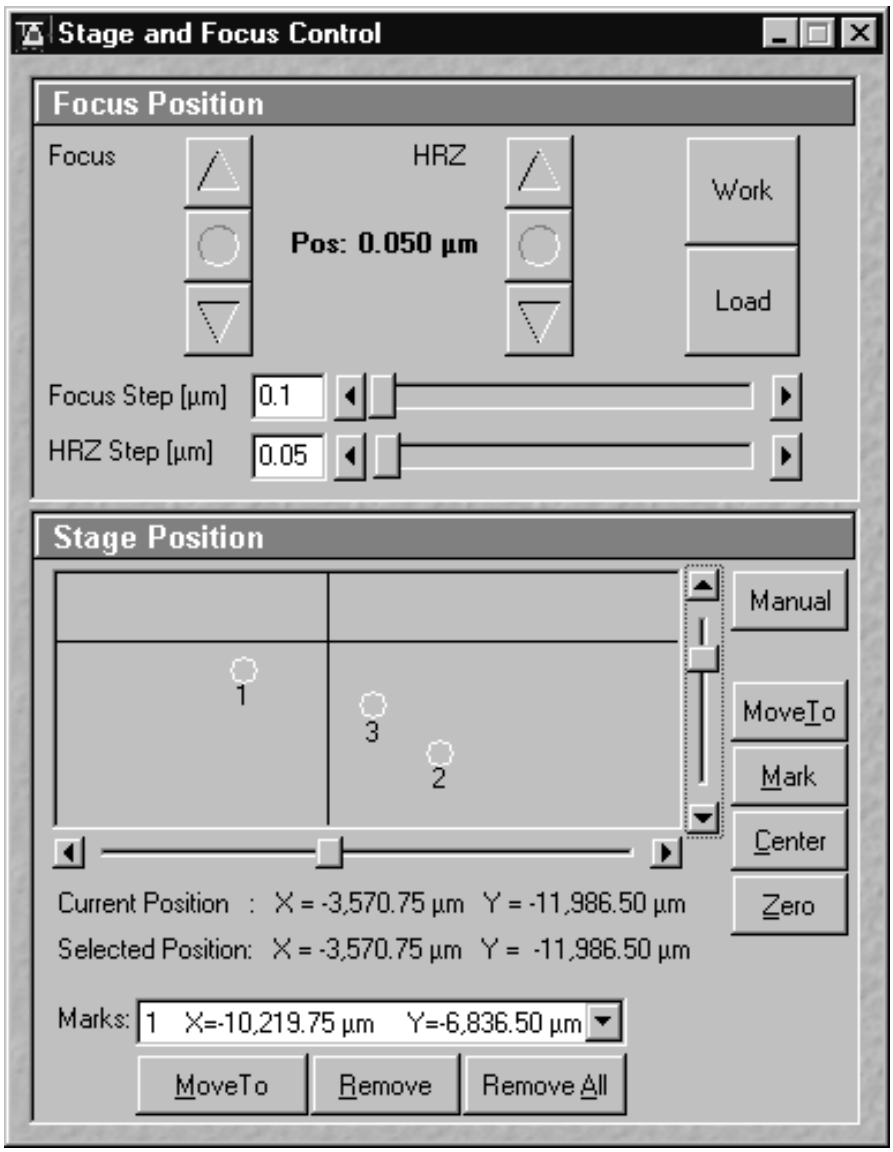


Fig. 4-175

This menu enables control of the scanning stage; a session-related MARK & FIND function is available to you.

The < **Stage Position** > window shows a symbolic specimen carrier in the left upper corner, with sliders for x and y.

The keys for moving to a position and mark it are on its right.

The **Current Position** and **Selected Position** display for X and Y is below.

Below that, you will find the table of marked positions and the possibility to activate and delete them.

Moving the scanning stage

The scanning stage can be moved using the joystick, or software-controlled using the sliders.

<Manual>

This button activates/deactivates the motor control of the stage and the joystick, if available.

If <Manual> is active, the scanning stage can be moved manually via the knurled screws. The <Move to> and <Center> function keys in <Stage Position> are without a function. The Current position is updated. You can zero the display via <ZERO> and mark manually set positions (<Mark>).

The scanning stage cannot be moved via the software or the joystick.

If <Manual> is deactivated, the scanning stage can be moved via the software or the joystick. All the functions of the "Stage Position" window are available.

<Move to>

Clicking on the <Move to> button moves the stage to the position marked in the symbolic specimen carrier. An area in the object slider is marked by clicking on the specimen carrier in the required area. The currently marked area is displayed by red crosslines in the specimen carrier. Their X/Y coordinates are displayed under **Selected Position**.

Already marked positions are shown in the specimen carrier as white circles with ordinal number and can be easily activated again by clicking and by triggering the Move command.

<Mark>

<Mark> allows the **Current Position** to be marked. This marked position is then stored in the Marks table in sequence. The marked position is shown on the specimen carrier with a circle and its ordinal number.

<Center>

Moves the stage to the current zero position.

<Zero>

Zeros the Current Position display and thus sets the currently set stage position to 0 in relation to X and Y. The already marked object areas thus receive new X and Y-coordinates.

<Current Position>

Current Position displays the currently set stage position in relation to the zero position.

<Selected Position>

Selected Position displays the coordinates of the position marked on the specimen carrier using the mouse.

<Marks> table

Clicking on the arrow keys displays the table of the session-related marked specimen areas. The table includes the ordinal number, the X-position and the Y-position.

<Move to>

Clicking on the <Move to> button activates the position selected before from the table.

<Remove>

The <Remove> command enables a selected position to be deleted from the table. The position then also disappears from the specimen carrier display.

CAUTION: The selected position is deleted, the position with the next number in sequence moves up one number.

<Remove all>

The < **Remove all** > command deletes all the entries marked in the current session.

Note:

The smallest step width displayed is:	0.1 µm
The smallest step width which can be set is:	0.25 µm
The smallest recommended step width is :	1.0 µm

4.12.6 Specification of Trigger-Interface LSM510

Application:

With the LSM510 Release 2.01 you can control various actions externally using Trigger-In or force external devices to work at a defined time depending on an action using Trigger-Out during time series. These actions are: Scan-Start/Stop, Bleach, Change of Scan-Interval, end of a countdown or even a mouse-click on a button.

Interface:

- Front plate Scanner-Interface (Scan-IF) inside
- Elektronic-Box (Scan-Control-Module) of LSM510:
- Connector 'User I/O', 26-pin shrinked SUB-D

Number:

- 4x Trigger-In, 4x Trigger-Out

Type/Voltage Range:

- TTL (HCMOS), 0.0 - 5.0 V

Load:

- In: 22 kOhm input impedance
- Out:± 4 mA

Trigger pulse description:

– Level detection:

Low level:	0.0 - 1.0 V
High level:	3.0 - 5.0 V

– Slew rate:

rising edge:	1 µs
falling edge:	1 µs

– Pulse width (always positive pulses/high level):

Trigger-In:	≥ 20 ms	(Speed 10 - 5)
	≥ 31 ms	(Speed 4)
	≥ 62 ms	(Speed 3)
	≥ 123 ms	(Speed 2)
	≥ 246 ms	(Speed 1)
Trigger-Out:	ca. 100 ms	

– Pulse frequency:

Trigger-In:	2x pulse width
Trigger-Out:	> pulse width

– Valid edge:

Trigger-In:	Rising edge
Trigger-Out:	Falling edge

Caution:

– Never apply more than 5 V or negative voltages to avoid any damage.

– In and outputs are not galvanically decoupled.

Therefore proper measures for galvanic decoupling external devices have to be taken (opto-coupler etc.).

– Do not connect no pins declared with ‘reserved’ (see table below). Otherwise at least the interface can be damaged.

Pin assignment:

No.	Name	Direction	Description
1	Trig1O	Out	Trigger Output #1
2	Trig2O	Out	Trigger Output #2
3	Trig3O	Out	Trigger Output #3
4	Trig4O	Out	Trigger Output #4
5...8	-	-	reserved
9	GND	-	Ground (0V)
10	Trig1I	In	Trigger Input #1
11	Trig2I	In	Trigger Input #2
12	Trig3I	In	Trigger Input #3
13	Trig4I	In	Trigger Input #4
14...17	-	-	reserved
18	GND	-	Ground (0V)
19...25	-	-	reserved
26	GND	-	Ground (0V)

4.12.7 Monitordiode

The monitordiode is placed in the excitation ray path of the LSM 510 behind the beam splitter combining the visible and the uv ray path and in front of the main beam splitter. Therefore it allows for checking the laser input in terms of power and noise. With the attenuation filter wheel in front of the diode it is possible to attenuate the laser power reaching the diode. It is not possible to select one line out of a few excitation wavelength to be detected by the diode.

To activate the diode as a detector do the following:

- Click on the corresponding button in the "Configuration Control" window of the LSM 510 software.

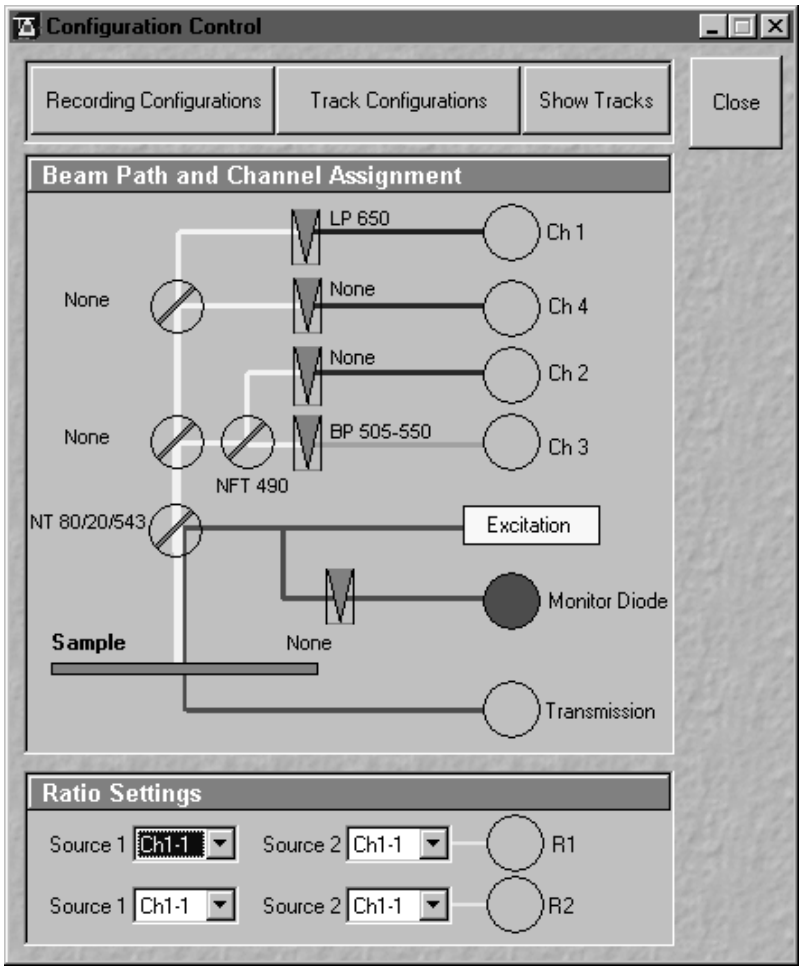


Fig. 4-176

- Choose either <Frame> or <Line> scan

- Change to the "Scan Control" window and press <Cont.>; the system will scan with the diode as a channel.
- Choose the right amplification of the signal obtained by using the special neutral density filters in front of the diode or / and by using the setting of the Amplifier Gain and Offset value. ("Scan Control" - <Channels> <ChM-1>).

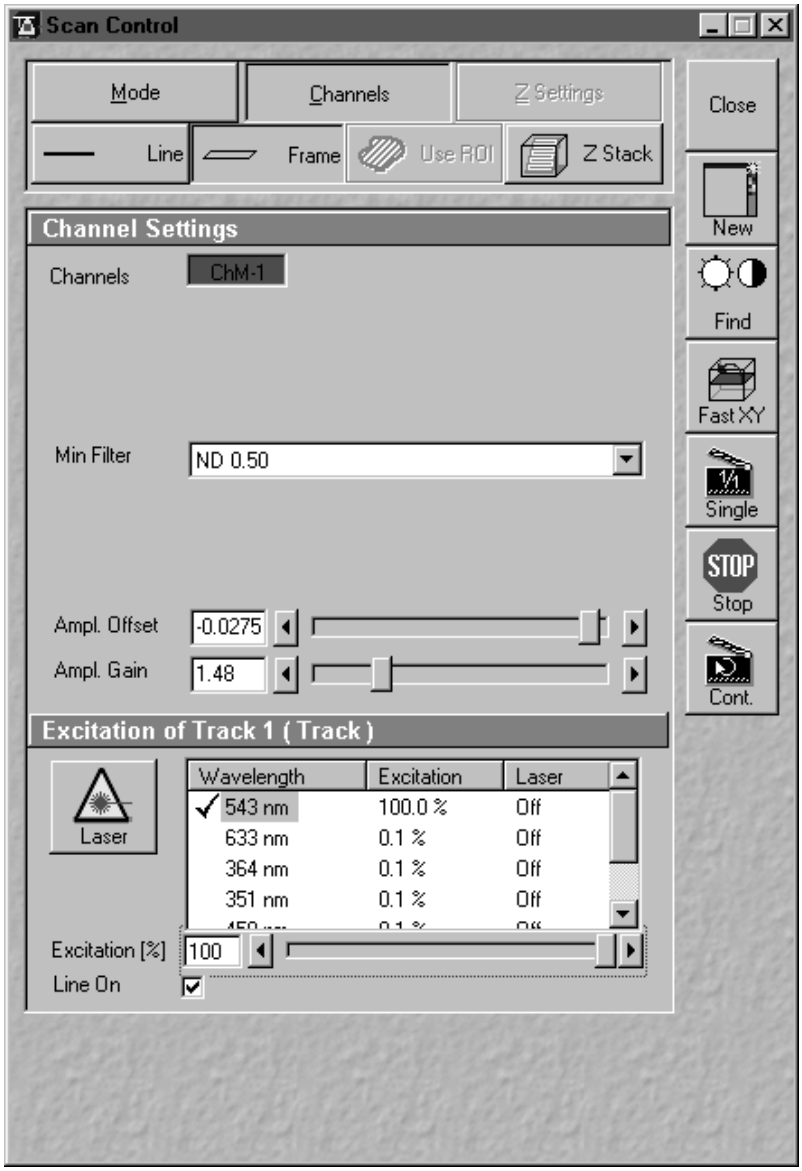


Fig. 4-177

Examples of application:

a) Checking the laser power

This function is not automized so far. To qualitatively measure the laser power the diode can be used in such a way, that the graylevel obtained in the **Line Scan** mode at a certain setting of the whole system is stored as a text overlay together with the image (manually done by user). As the diode setting (Ampl. Gain, Ampl. Offset, ND filter) is stored together with the image, the setting is automatically reloaded when using the <REUSE> button. If deviations can be observed it is easy to set the laser power to the old value by means of the AOTF transmission.

b) Noise Reduction by Ratio

In contrary to the PMT signal, the signal of the monitor diode <is not modulated by any specimen information. Thus it can be used to ratio the PMT signal to get rid of the laser noise (due to any laser as a physical fact) and thereby improve the signal to noise ratio of the fluorescence or reflectance image. The major condition which has to be fullfilled to use the monitor diode for this purpose, is, that the dominating source of noise is laser noise. The signal of the monitor diode will always be dominated by laser noise (independent of the power set at the laser, or the transmission set at the AOTF), whereas the dominating source of noise in the PMT signal can also be the shot noise of light (shot noise especially occurs in low light fluorescence application; as rule of thumb it can be noted, that the shot noise is limiting the signal to noise ratio, if the PMT voltage has to be set to a value > 400V).

Note: Any kind of noise which can not be observed in both channels at a time will be amplified and not reduced by the ratio process. Low or high frequency laser noise is the only source of noise which is correlated in the PMT signal and the signal of the monitor diode.

Using the HeNe lasers, noise is extremly low, so that in all applications, if, the shot noise of light will reduce the signal to noise will reduce the signal to noise ratio of the image. Low or high frequency laser noise is mainly introduced, if the Ar, ArKr lasers are used at a tube current lower than 8A (Ar-Vis, ArKr) or 20 A (Ar-UV) respectively.

To use the monitordiode for ratio application the following steps have to be done:

- Activate the ratio channel R1 or R2 in <Ratio Settings> of the menu <Configuration Control> in addition to the monitor diode channel (ChM-1) and one PMT channel.
- Choose the appropriate PMT channel as source 1 in <Ratio settings> and ChM-1 as source 2. If this numbering is changed (inverted) the ratio image will show a invertation of gray levels if compared to the PMT image.

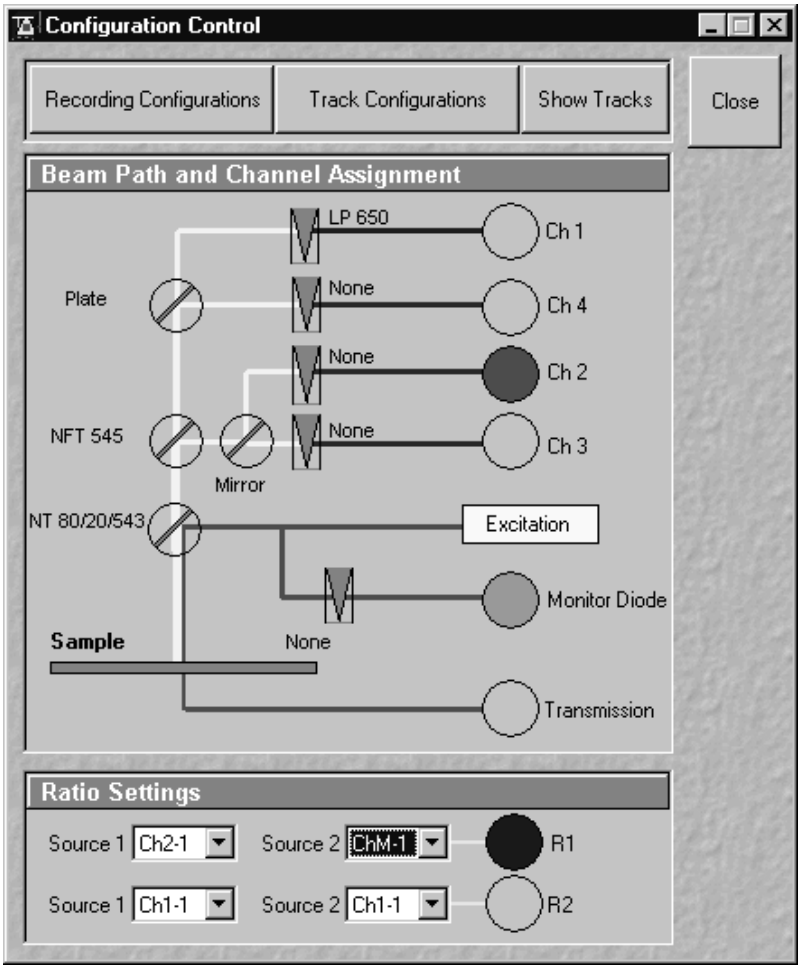


Fig. 4-178

☞ It is not possible to do the ratio between an on-line ratio image generated with two PMT channels (as in ion-concentration sensitive ratio imaging) and the signal of the monitor diode.

The following image is an example of the reduction of correlated noise. The low frequency noise has been generated artificially.

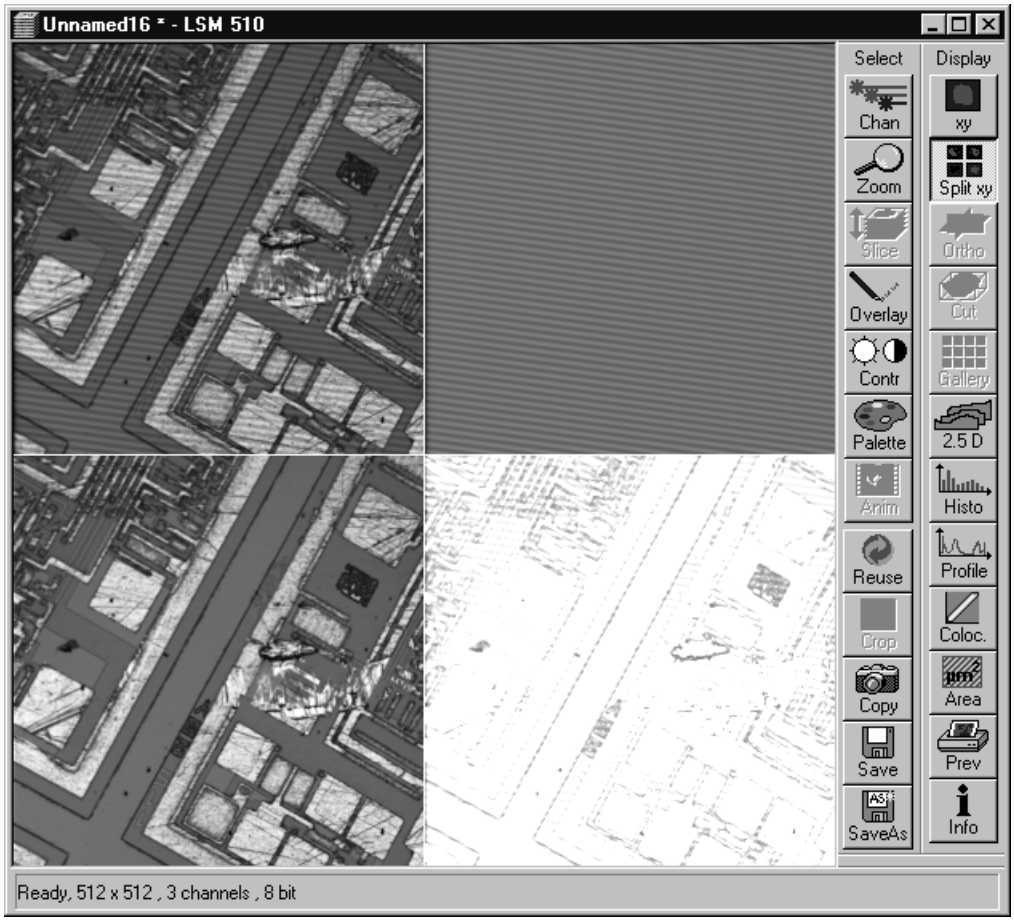


Fig. 4-179

The image in the upper left corner shows the PMT image plus noise, the image beneath this (upper right corner) shows the signal of the diode expanded to 512x512 pixels (noise without object information). The two images below show the ratio of the PMT and diode signal (left) and the sum of all signals (right). The sum-image does not contain any information and can therefore be neglected.

To get a ratio image like the one shown here the setting of: Detector Gain, Amplifier Gain , Amplifier Offset of the PMT channel, Gain and Offset of the diode channel, Gain and Offset of the ratio channel has to be set in the correct way.

Each of the parameters summarized effects either the amplification of the ratio image, or the contrast of the ratio image, or the quality of the noise reduction.

The single steps to find the right setting of all the parameters to be set are listed in the following:

- Activate the <Range Indicator>
- Adjustment of **Amplifier Offset**: the Offset of the PMT channel and diode channel have to fit to each other to guarantee the best noise reduction.

The best way to do the adjustment is the following:

- Choose different colors in <Configuration Scan> for PMT and diode channel
- Activate <Line Scan>
- Switch off all laser lines in the <Excitation> window
- Activate <Cont>
- Set values for Ampl. Gain to 1 in each channel
- set the lines visible to the same level as close to the ground level as possible; the values you find for the Offset in each channel should be negative.

A final adjustment of the offset adjustment is done by visually evaluating the noise reduction in the ratio image. As the Offset value of the PMT channel influences the range setting of the ratio image much less, than the Offset value of the diode channel the fine tuning should be done with the PMT offset if necessary.

☞ As told before, the calculation of the ratio image is very sensitive to different signal offsets in the two channels used. As the offset is influenced by the scan speed as well as by the Amplifier Gain used, the offset calibration is not valid any more, if the scan speed is changed, or the Ampl. Gain is set to a new value respectively. In most cases a new fine tuning is necessary. If this doesn't work the complete calibration process has to be repeated.

Another possibility to calibrate the offset values is to set the values to -0.1 as default for both channels, then do steps 3 and 4 and finally do the adjustment of the noise reduction by varying the PMT offset value.

If the ratio application is used and the offset has been set to the best reduction of noise in the ratio image it is not allowed to change the offset of the PMT channel to change the reduction of background fluorescence for example. This can be done only if the diode offset is corrected afterwards.

Adjustment of Detector Gain

The Gain of the PMT should be set with the help of the range indicator function. No 'red ' and no 'blue' pixels should occur in the image of the PMT.

Amplifier Gain

The diode signal is set to the right range (graylevel between 50 and 200 - 8bit image / 750 and 3500 - 12 bit image) with the help of gray filters and amplifier gain. The use of a lower filter density should be priorized against the use of a high gain value. The value of the amplifier gain of both channels (PMT and diode) should be set to one if possible. Because of a, parallel to the gain factor, increasing amplifier noise, a gain value of more than 2 should be avoided. The most important thing is, that no pixels are below the zero level and beyond the maximum range respectively.

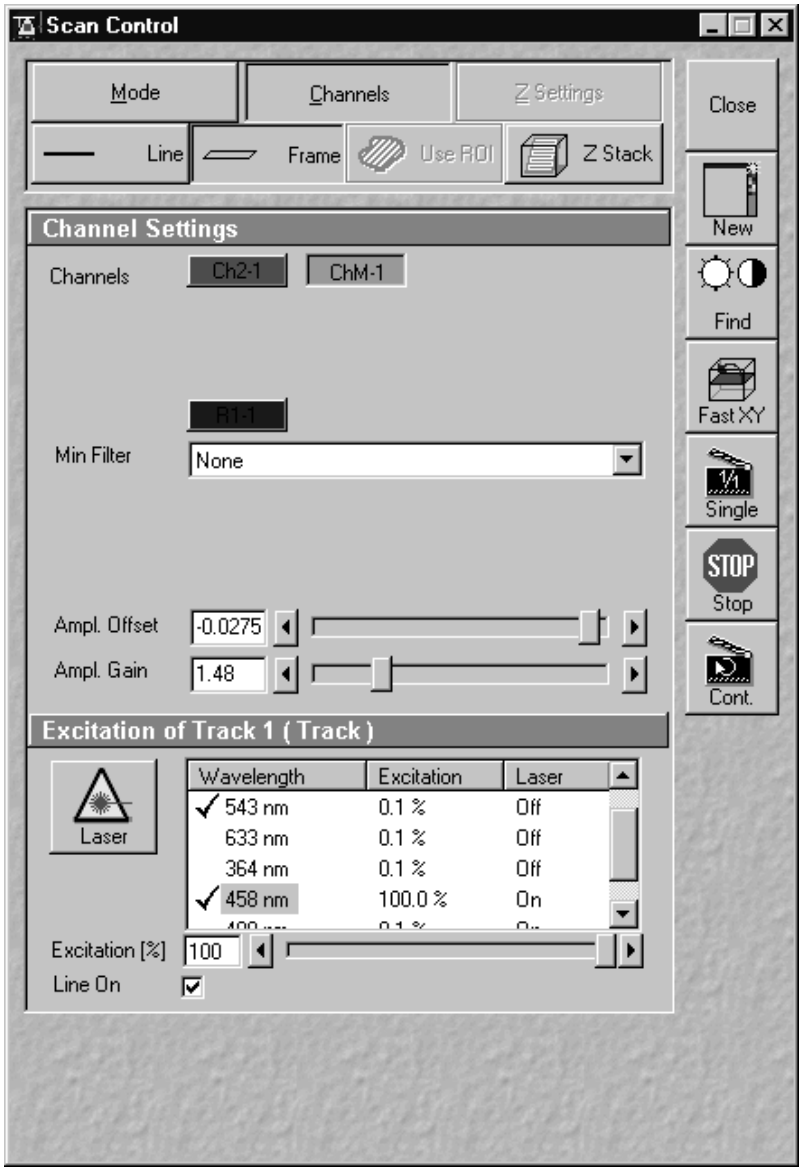


Fig. 4-180

Gain and offset in Ratio channel

If the setting of the PMT channel is finished, the range of the ratio channel is adjusted by the parameters in the corresponding formalism. There are three types of formulas offered, when the button <R1-1> is pushed. The only formula needed for a ratio image with the monitor-diode is type 1:

$$\frac{S1+n}{S2+m} * x + y$$

The values for n and m have to be zero, as well as the value for y. Any deviation from zero will decrease the contrast of the ratio image.

Only the value of x shall be influenced by the user. Dependent on the choice of data depth (8 or 12 bit), x is between 0 and 256 (8bit) or between 0 and 4096 (12bit).

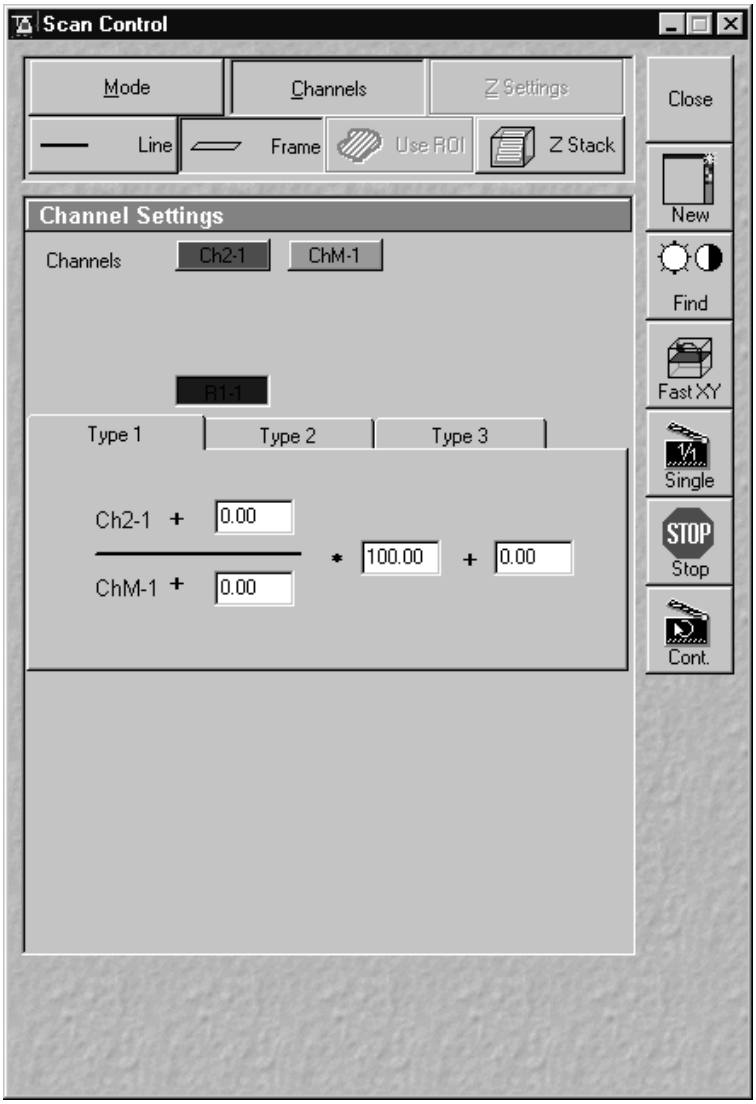


Fig. 4-181

Default settings are 150 and 3000 respectively. With the help of the range indicator the default value is changed until there is no pixel overflow anymore (‘red pixels’)

☞ Any new value can be set by hand typing and pressing the <ENTER> key while the scan is running.

Any change in the setting parameters of PMT and diode signal will make a new Gain x in the ratio formula necessary.

If the adjustment of all parameters is finished only the ratio image can be scanned respectively displayed by switching off the PMT channel and the diode channel in the "Configuration Control" window and leaving only the Ratio Channel be turned on. As a result only the ratio image is displayed; which can still be influenced by the settings in PMT and diode channel.

CHAPTER 5 3D FOR LSM 510

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5 3D FOR LSM 510

5.1 Overview and Explanations

5.1.1 The Image Sequence

The "3D for LSM" handles image sequences generated by the Zeiss LSM software. This can be three-dimensional image data or a time sequence of two-dimensional images (slices). Each slice (as well as the sequence) can consist of up to eight channels. An image sequence consists of a series of individual (2D) images and has a name that designates the entire sequence. In general an image sequence is handled as a single object in the system. Individual channels or slices can be addressed.

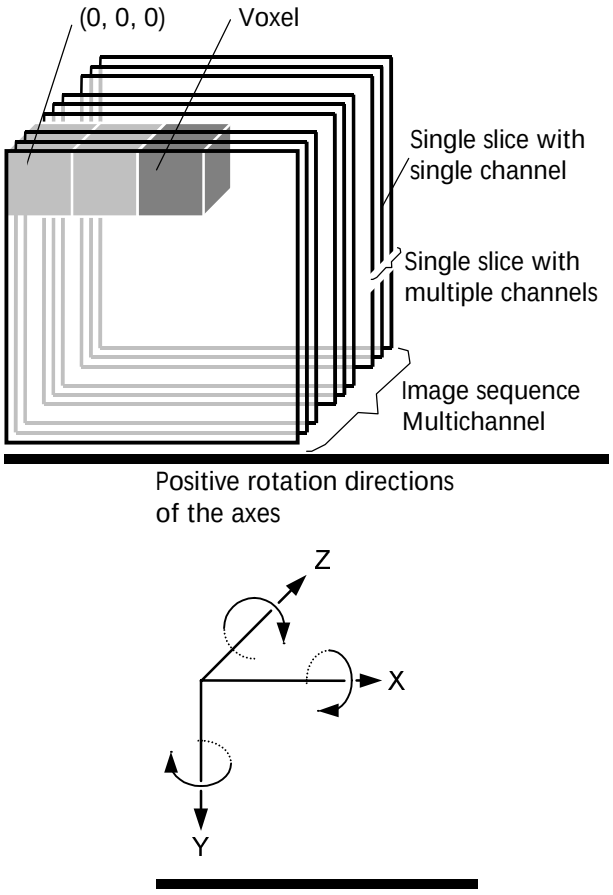


Fig. 5-1

The following terms and definitions apply for the "3D for LSM" software.

- An image sequence is a number of individual sequential images (usually called slices in the dialog boxes), the spacing between which is equal.
- Image sequences can contain up to 12 bit of image data (per channel).
- A sequence (slice) can consist of up to eight channels.
- The maximum size of an image sequence is limited by the provided memory of the operating system.
- A voxel is the smallest element of an image sequence (the equivalent of a pixel in a 2D image). All voxels in a given image sequence are the same size.
- The coordinate system originates in the left upper front corner of the image sequence. This point has the coordinates 0, 0, 0.
- All angles are positive for rotations to the right in the direction of the positive coordinate axis (right-handed coordinate system).
- A slice is an individual image in a sequence of images. The numbering of the slices starts with "1".

Image sequences can consist of several channels. Most functions and the **Display window** are providing buttons to select all or a subset of channels stored in the selected image sequence. The **Output** image sequence will only get those channels which are selected on the input side. The button selects all channels in the image sequence to be used clicking with the left mouse button on it.

Clicking with the left mouse button on any of the number buttons toggles the state of this single channel.

Clicking with the right mouse button on any of the number buttons selects this single channel exclusively. All other channels are deselected.

5.1.2 The Image Properties

Every image sequence has its own set of properties. They contain the scaling and the scaling units. The scaling and its units are required for 3D reconstruction and measurement. If a sequence of LSM-TIFF images is read in, the image properties are loaded automatically from the file header and allocated to the image properties of the new image sequence.

5.1.3 Memory Usage

All images shown in the **Gallery** are currently loaded in the system memory of the operating system. Some functions need additional temporarily used memory during their execution.

If the memory is running low delete some images from the **Gallery**. If the images are needed afterwards they must be saved to disk first. Normally all functions produce a new result (output) image sequence. In order to save some memory, other image sequences currently presented in the **Gallery** can be selected as result position. The output image is overwritten by entry execution of a function.

5.2 User Interface

5.2.1 Introduction

This section describes the following main components of the system:

Main window — **Main window** with the **Menu**, the **Tool bar** and **Gallery**. All general system functions are located here.

Gallery — Normally several images are required in order to accomplish a particular task. These images are displayed in reduced size to provide an overview and facilitate selection. This area is located just below the **Tool bar**.

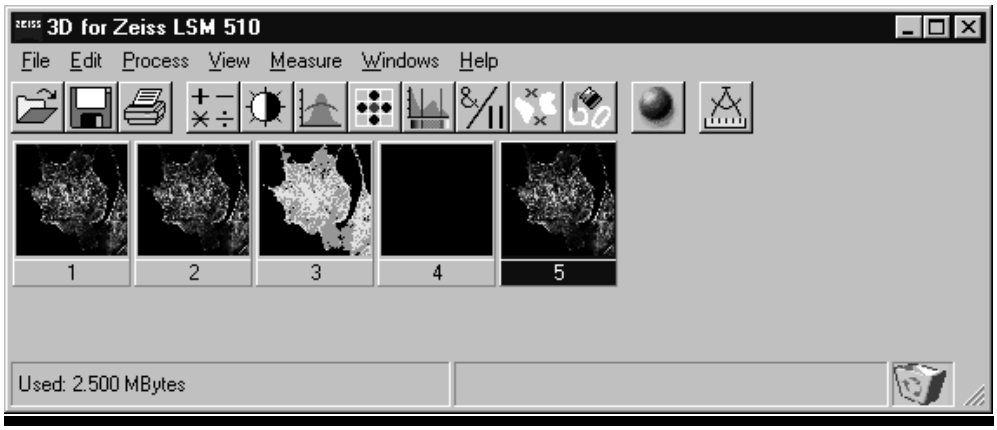


Fig. 5-2

Tool bar — This menu shows all image processing functions.

Display window This window is used to display image sequences.

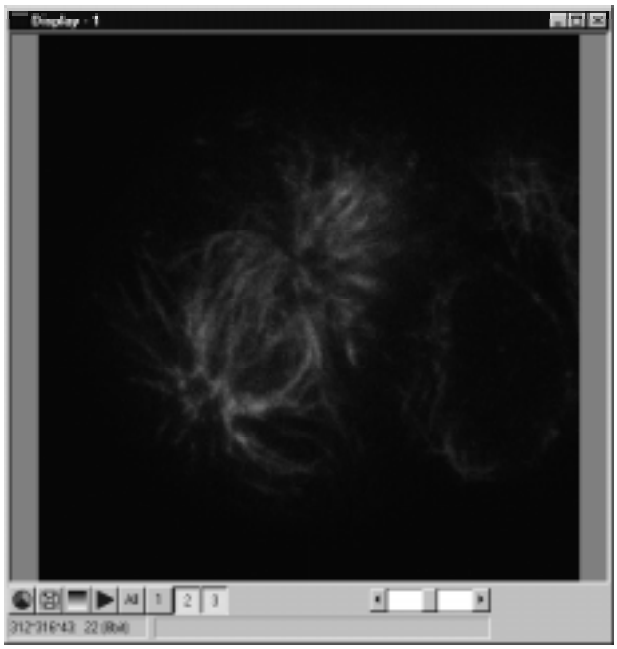


Fig. 5-3 Display window

Dialog boxes All dialog boxes provide three buttons. Pressing the **OK** button executes the function with the defined parameters and closes the dialog window. Selecting the **Cancel** button does not execute the function, restores the parameters, and closes the dialog window. Pressing the **Apply** button executes the function with the defined parameters; the dialog window will stay opened.

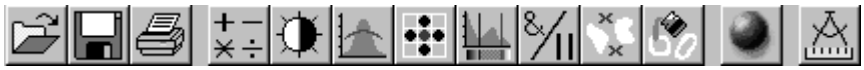
5.2.2 Main Window

The **Main window** includes:

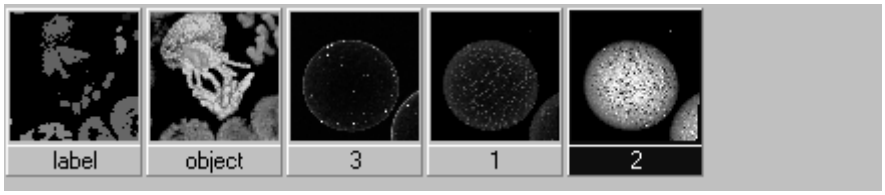
the **Menu**



the **Tool bar**



and the **Gallery**



File Menu



Open Image Opens a file selector dialog to load an image sequence.



Save Image As Opens a file selector to save an image or image sequence.

Save Display As Saves the currently shown contents of the Display window as a single colour image.



Print The printer parameters can be set with this tool. The standard Windows printer dialog is opened.

Exit Terminates the application.

Edit Menu

Copy	Copies the contents of the **Display window** to the clipboard.
Edit Channels	Allows to add or to remove channels to a single or multichannel image.
Delete All Images	Deletes all images and image sequences from the memory.

Process Menu


Arithmetics Adds or subtracts the grey values of two image sequences (**Add**, **Subtract**).


Contrast Enhances the contrast and brightness of an image sequence (**Interactive**, **Automatic**, **Linearize**).


Smooth Smoothes an image sequence.


Morphology Performs morphological operations on image sequences (**Erode**, **Dilate**, **Open**, **Close**).


Segment Segmentates an image sequence to propose measurement (**Interactive**, **Automatic**).


Boolean Combines two image sequences by Boolean operations (**And**, **Or**, **Not**, **Xor**, **Mask**).


Scrap Selects or deletes objects of a defined size.


Fill Holes Fills holes in objects.

View Menu

Set Channel Colour	The colour and the weight of the single channels can be defined.
Properties	The properties of the image (e.g. scaling, use laser etc.) are displayed.
 **Render**	Calculates 3D reconstructions of an image sequence (Surface, Alpha).

Measurement Menu

 **Automatic Object**	Measures geometrical and densitometrical features (**General**, **Object Features**, **Volume Features**, **Condition**).

Windows Menu

Arrange All	Arranges the windows automatically.
Display	The current image is displayed in this window.

Help Menu

Content	Opens the help for the software.
About 3D for LSM	Displays status and release message of the software.

Tool Bar

This bar provides buttons with iconized images of nearly all functions. Clicking on one of the buttons will open a dialog window to define the function parameters. Selecting an entry from the menu alternatively can activate the same functions. Placing the cursor on a tool bar button will show a short description, if the window is activated.

Gallery

The **Gallery** is used as an overview of the images available in memory and their contents. It is located just below the **Tool bar**. Each small image represents a sequence. The middle slice of each image sequence is shown. The status bar of each image shows the name. The name might be a number or a string.

Every image sequence has its own channel colour assignment (see **Display window**). When an image is copied the channel colour assignment is copied too. Drag and drop techniques can be applied to copy images or define the function parameters **Input** and **Output** using the **Gallery** thumbnails.

- Position the cursor on an image in the **Gallery**.
- Press the left mouse button.
- Hold the mouse button down and move the mouse to the destination position.
- At the destination release the left mouse button, the destination image will be overwritten.

To delete an image, drag it, move it to the wastebasket, and drop it.

5.2.3 Display Window

This window is used to display an image sequence, regardless of size or type. To show multiple channel sequences each channel could have its own base colour. The user can set these colours and the weighting for each channel by pressing the corresponding button  at the bottom of the window. To display a different image or image sequence, it can be dragged from the **Gallery** and dropped to the **Display window**.

The image can be displayed in full size (one pixel on the screen represents one pixel of the image) or in a zoomed size. To zoom the display view click and hold down the right mouse button on the window border and resize the window. The aspect ratio of the image will not be changed. Clicking on the button  resets the **Display window** to a full size view of the image (see above).

The title bar shows the currently displayed sequence name. The status bar displays the size of the current sequence and the selected slice on the left. On the right the cursor position within the window and the corresponding intensity (grey) value of each channel is shown.

The **Display window** can be closed without any effect to the image processing functions. If no **Display window** is opened select the entry **Display** in the **Window menu**.

The scroll bar at the lower right of the window enables to show the images in a sequence. The range reaches from one to the maximum slice provided by the current sequence.

To start the automatic animation of an image sequence start the Player tool by clicking on the button  . The colour selection for the channels can be activated by clicking on the button  . A colour image can be displayed as a grey value image by clicking on the button  .

Player

This function plays back the sequential images of an image sequence.

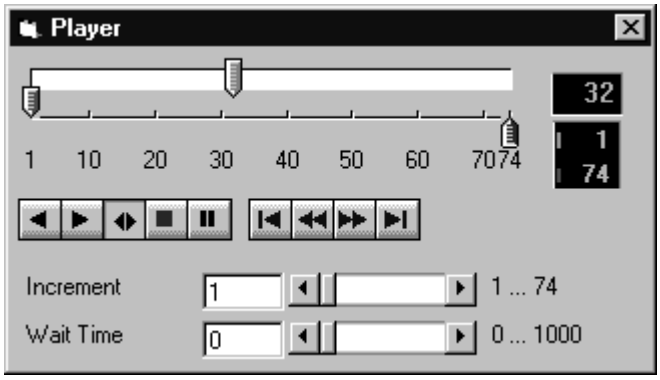


Fig. 5-4

The image sequence is displayed in the **Display window**. The display process is working as a background task; other functions can be executed while the player is running. There are several ways to stop the player:

by closing the player window

by pushing the red Stop button of the player window (the window remains open)

by closing the image window.

The **Increment** parameter specifies whether each sequential image (1) should be displayed or whether some sequential images should be skipped during display. The value 2 skips one image for every sequential image displayed, in other words, it displays only every second image.

The parameter **Wait Time** states the delay in milliseconds between two successive sequential images. The maximum display speed depends mainly on the hardware. The sequential images are always displayed in their entirety, regardless of the set delay.

Control Element of the Player

The three arrow shaped controls on the scale show the start slice and the currently displayed sequential image. The values (positions) can be changed using the mouse. Press and hold the left mouse button and move the pointer to the desired position. The set values are shown in the numerical windows at right.

 Start slice

 Currently displayed sequential image

 End slice

The buttons in the left group start and stop playback of an image sequence.

 Reverse playback

 Forward playback

 Play forward and then backward again (jojo)

 Stop playback

 Pause playback

The buttons in the middle group control the settings of the current sequential image.

 Reset to start slice.

 Single step backward (1 sequential image each regardless of Increment).

 Single step forward (1 sequential image each regardless of Increment).

 Set to end slice.

Increment Image increment.

Wait Time Displays delay between two images (in milliseconds).

Set Channel Colour

This function sets the colour and weight for the channels.

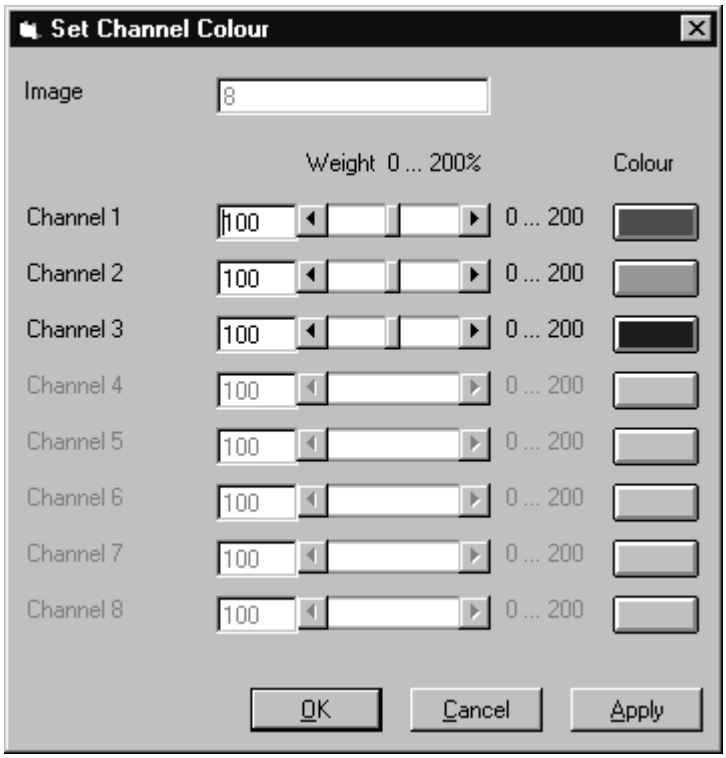


Fig. 5-5

Each image sequence can get its own colour definitions. All functions will inherit the colour definition from the **Input** sequence to the **Output** sequence. By default the colours are set to 100 % weighting and the pure base colours (red, green, blue) are defined.

The weight can be any value between 0 % and 200 %. The colour can be redefined by clicking on the coloured button on the right of the dialog. The standard Windows colour selection dialog is opened. The solution is done by clicking on one of the colours or by entering appropriate numbers in the corresponding edit boxes.

Pressing the **OK** button will close the colour selection dialog and update the **Display window** immediately.

Only those channels, which are available in the image sequence, can be defined.

Parameters:

Image	Image sequence to edit
Weight	Colour weighting for each channel
Colour	Base colour for each channel

5.3 Functions

5.3.1 Functions in the File Menu

Open Image

This function reads a Zeiss LSM 510 (*.lsm), Zeiss LSM TIFF (*000.tif) or Carl Zeiss Vision (*0.img) image sequence from a disk or network drive.

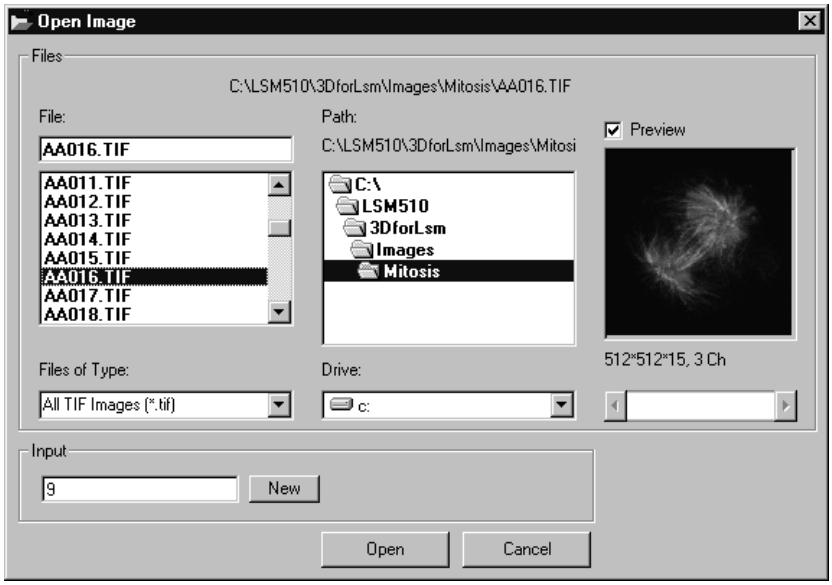


Fig. 5-6

The individual files of a Zeiss TIFF image sequence are read and saved as an image sequence in image memory. In addition, the image properties are read out of the TIFF files and allocated to the image sequence **Input**.

The directories of the current drive are listed in the **Directories** list box. Use the **Drives** list box to choose a different drive.

In case of choosing the TIFF-format in the **Files of Type** box, three number characters are always expected before the dot in the filename extension. The first number must be 000 at the end of the filename. From a complete sequence only this file is listed in the dialog, if "LSM TIF Images (*000.tif)" is selected in the **Files of Type** box. To view all TIFF files "All TIF Images (*.tif)" in the **Files of Type** box must be selected. This selection enables to start with a different file than with the very first (named *000.tif) at the end of the filenames three number digits.

Currently the Carl Zeiss Vision file format "KE Images (*0.img)" is supported. Two files per channel are saved.

Carl Zeiss Vision image sequences must have a number digit at the end of the base filename. They are used to indicate the different channels in a multichannel sequence. The numbering starts with zero (0). If a sequence is saved in the Carl Zeiss Vision format the numbers are generated automatically. To load such an image sequence "KE Images (*0.img)" in the **Files of Type** box must be selected.

The window incorporates the usual file selection controls. The bottom half displays a selection of the image properties that are stored in the image sequence.

Parameters:

BaseName Base name of the TIFF files (image sequence) to be loaded. Only the letters before the first number are stated.

Input Name of the resulting image in which the image sequence will be saved.

Save Image As

This function saves an image or image sequence to disk or network drive.

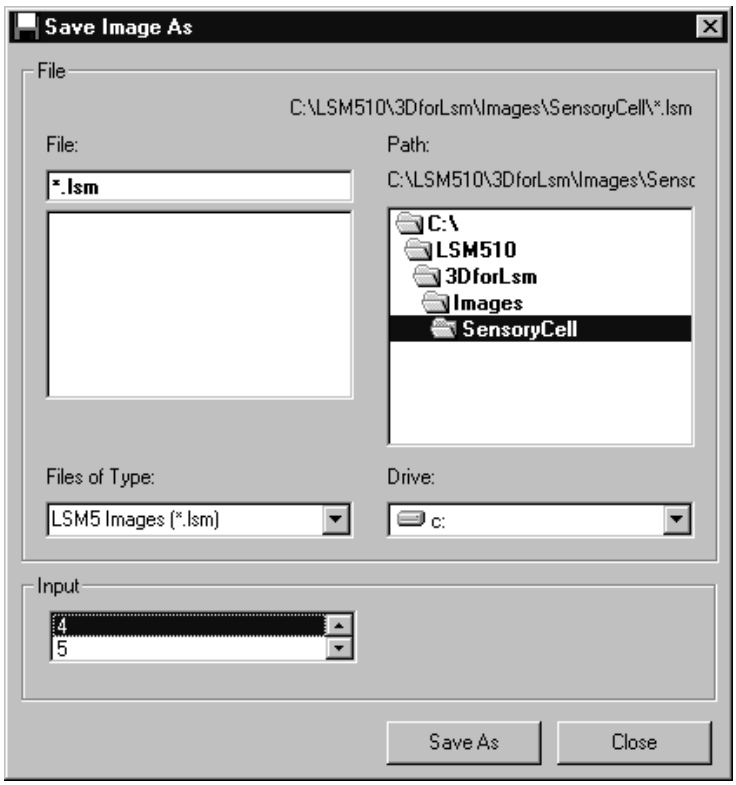


Fig. 5-7

All the files in the current directory that have the selected image format are listed in the **File Name** list box.

The directories of the current drive are listed in the **Directories** list box. Use the **Drives** list box to choose a different drive.

Use the list box **Files of Type** to select the image format. Currently the LSM 510 image format (*.lsm) and the Carl Zeiss Vision file format "KE Images (*0.img)" is supported.

By choosing the Carl Zeiss Vision file format "KE Images (*0.img)", two files per channel are saved. On one hand the Carl Zeiss Vision type image sequence file, on the other hand the file with the image properties. One pair of files is written per channel. They are numbered automatically, starting with zero. A one number digit is added to the end of the filenames. The two files share the same filename but have different filename extensions (*.img and *.3d).

The content of the **Gallery** is shown in the **Input** section. The selection of the sequence to save is done by highlighting one of the provided names or by drag and drop from the **Gallery**.

Parameters:

Input Name of the image sequence to be saved

Filename Name of the file to be used on disk

Save Display As

This function saves the current **Display window** contents to a disk or network drive.

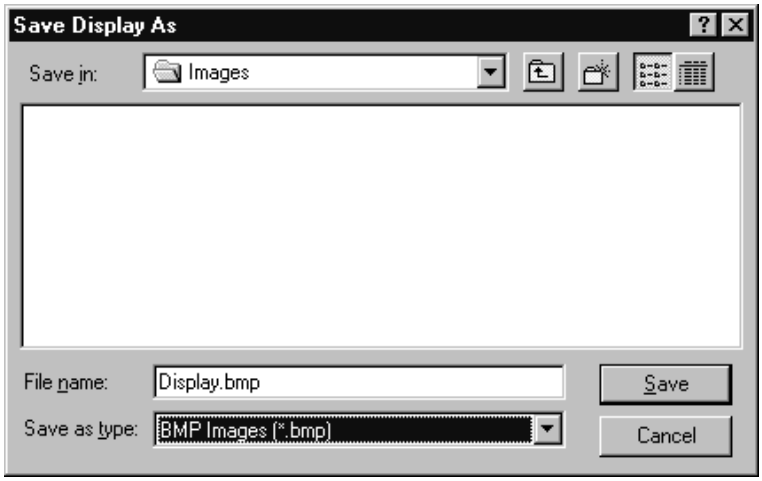


Fig. 5-8

Before the execution of this function any image or image sequence can be selected to be displayed. From a multichannel sequence any channel status (on or off) combination can be defined. The colours of the shown channels can be set with the function **Set Channel Colour**.

The current zoom factor of the **Display window** is not taken into account, the image is saved without any zoom.

The image is saved as a true colour image with 24-bit resolution. From the **Save as Type** list box one of the provided formats can be selected.

Parameters:

None

Print

This function prints the current **Display window** contents.

The standard Windows print dialog is opened.

Before the execution of this function any image or image sequence can be selected to be displayed. From a multichannel sequence any channel status (on or off) combination can be defined. The colours of the shown channels can be set with the function **Set Channel Colour**.

Parameters:

None

Exit

This function terminates the application completely.

All images and image sequences shown in the **Gallery** will be deleted from the memory. Save those images which might be used for any further processing.

Parameters:

None

5.3.2 Functions in the Edit Menu

Copy

This function copies the current **Display window** contents to the clipboard. No dialog is shown.

Before the execution of this function any image or image sequence can be selected to be displayed. From a multichannel sequence any channel status (on or off) combination can be defined. The colours of the shown channels can be set with the function **Set Channel Colour**.

The current zoom factor of the **Display window** is not taken into account; the image is copied without any zoom.

The image is copied as a true colour image with 24-bit resolution. Afterwards the contents can be pasted to any other Windows application.

Parameters:

None

Edit Channels

This function allows to add or to remove channels to a single or multichannel image.

On the **Add Channel** tab sheet the channels of (different) **Input** sequences can be defined to add (combine) channels to an **Output** sequence.

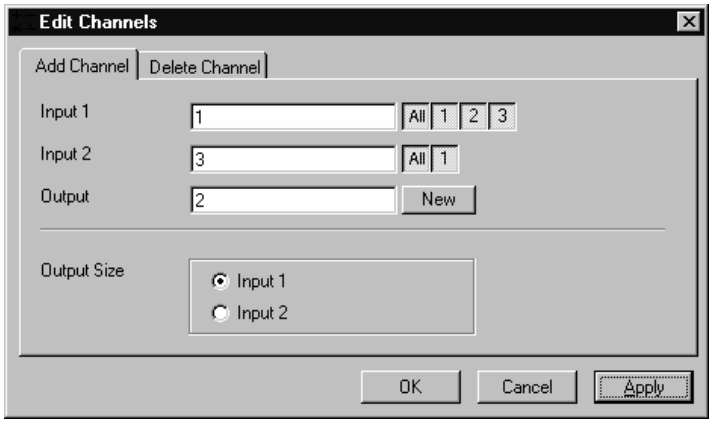


Fig. 5-9

This operation is useful to add a segmented channel (or any other result of a function) to the original image sequence. The selected channels of **Input 1** and **Input 2** are copied to **Output**. The maximum number of channels in an image sequence is eight.

If the image sequences do not have the same extents **Output Size** defines which input is taken as a reference. This selection also defines the properties for scaling and units in the output image sequences.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Output image sequence
Output size	Defines source image sequence for size, scaling, and units

On the **Delete Channel** tab sheet channels of the **Input 1** image sequence can be selected to delete channels.

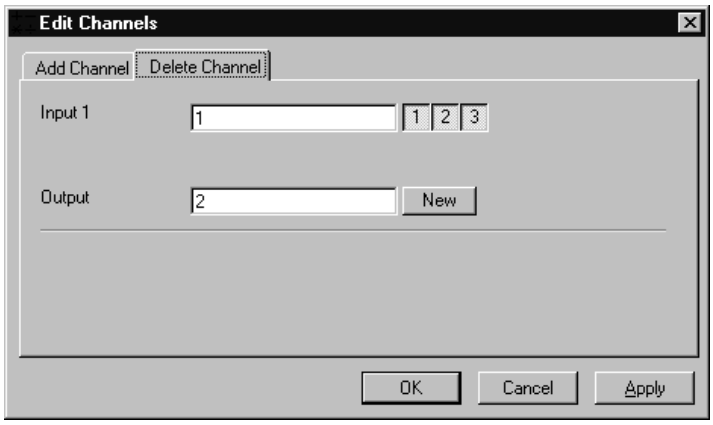


Fig. 5-10

This operation might save time and memory for further processing if not all channels are needed.

Only the selected channels of **Input 1** are copied to **Output**.

Parameters:

Input 1 Input image sequence

Output Output image sequence

Delete All Images

This function deletes all images and image sequences from the memory (**Gallery**).

The function is used whenever a completely new image sequence should be processed. In order to drop the images item by item to the wastebasket all of them can be deleted by a single function.

If any image or image sequence is needed for further use save them first.

Parameters:

None

5.3.3 Functions in the Process Menu

Arithmetics - Add

This function adds two image sequences.

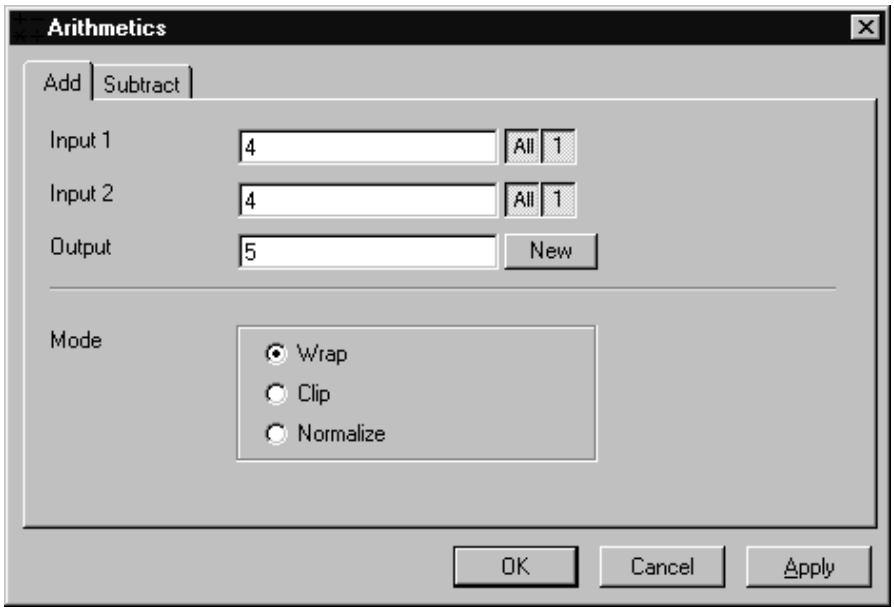


Fig. 5-11

The **Add** tab sheet of the **Arithmetics** dialog window must be selected.

If one or both input sequences are multichannel sequence, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

This function adds the two image sequences **Input 1** and **Input 2** voxel by voxel and generates the image sequence **Output**. Note that a resulting grey value may be greater than 255 (4095). The parameter **Mode** determines how a range overflow is handled:

1 - Wrap	No normalization - the grey values are displayed modulo 256 (4096). If the result is greater than 255 (4095), the value 256 (4096) is subtracted from it.
2 - Clip	Grey values which exceed 255 (4095) are replaced with 255 (4095).
3 - Normalize	The resulting grey value range is scaled to the range 0...255 (0...4095).

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Output image sequence
Mode	1 - Wrap 2 - Clip 3 - Normalize

Arithmetics - Subtract

This function subtracts two image sequences.

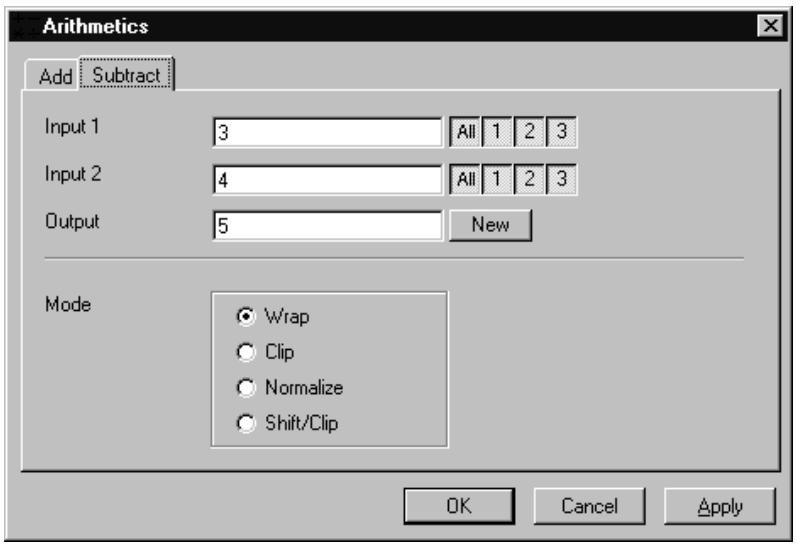


Fig. 5-12

The **Subtract** tab sheet of the **Arithmetics** dialog window must be selected.

If one or both input sequences are multichannel sequence, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

This function subtracts the two image sequences **Input 1** and **Input 2** voxel by voxel and generates the image sequence **Output**. Note that a resulting grey value may be less than 0. The parameter **Mode** determines how a range overflow (negative values) is handled.

1 - Wrap	No normalization - the grey values are displayed modulo 256 (4096). If the result is less than 0, the value 256 (4096) is added to it.
2 - Clip	Negative values are set to 0.
3 - Normalize	The resulting grey value range is scaled to the range 0...255 (0...4095).
4 - Shift/Clip	128 (2048) is added to the difference, then negative values are set to 0. Values greater than 255 (4095) are set to 255 (4095).

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Output image sequence
Mode	1 - Wrap 2 - Clip 3 - Normalize 4 - Shift/Clip

Contrast - Interactive

This function allows interactive changes of the contrast of an image sequence.

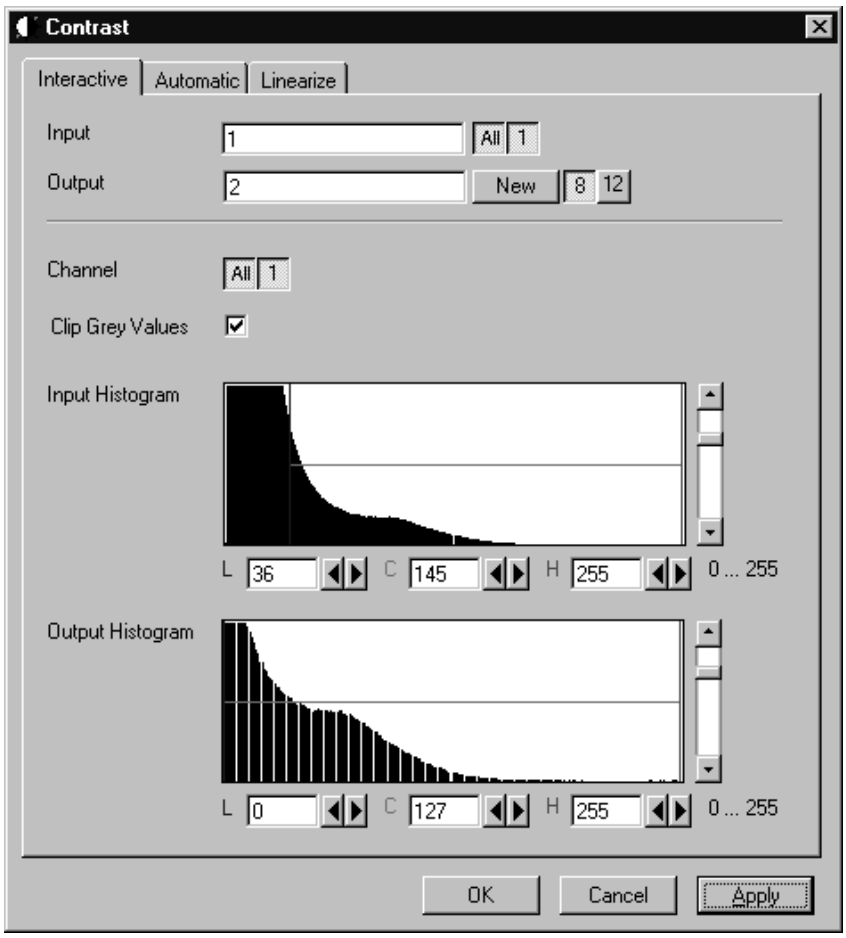


Fig. 5-13

The **Interactive** tab sheet of the **Contrast** dialog window must be selected.

A grey value range of the **Input** image sequence is scaled to another range in the **Output** image sequence. Both ranges can be edited interactively. This function is used to achieve a better view of an image sequence, or to scale a range of grey values to single value for a special coding in an image sequence. The function does not improve the result of the linear segmentation function **Segment**.

Input indicates the sequence to enhance. If it is a multichannel sequence, a single channel, all channels, or any number can be selected. The **Input** histogram shows the grey value distribution of the selected channels of the **Input** image sequence.

Output defines the name of the result sequence. It will get only those channels which are chosen by the **Input** parameter. The buttons labeled with 8 and 12 define the grey value (intensity) resolution in bit. Normally the result will get the same resolution as the **Input** sequence. A change will be needed if image sequences with different resolutions should be combined. Rising the grey value range to 12 bit will not enhance the display quality or measurement accuracy. The smooth and morphology functions will produce results with finer gradations.

If **Clip Grey Values** is selected, the output grey values are clipped to the **Low (L)** and **High (H)** values. If **Clip Grey Values** is not selected, output grey values beyond the **Low** and **High** value range are possible.

The **Output** histogram shows the resulting histogram. The horizontal axis represents the grey values from 0 to the maximum, which is either 255 or 4095, depending whether the input is 8 bit or 12 bit. The vertical axis represents the pixel count. The selected range is marked by the borderlines in the histogram. The blue line or **L** indicates the lower boundary, the red line or **H** the upper one, **C** indicates the center of the range.

There are three ways to change the range: clicking and dragging the borderlines with the mouse.

Entering a new value in the appropriate text boxes, clicking on the buttons  or using the arrow keys from the keyboard. To alter the values within the histogram move the mouse pointer over one of the three coloured lines until the shape changes. Press and hold the left mouse button to move the line to a new position. To change the values with the arrow keys click once into the histogram. Using the left or right arrow key by its own will move the whole range. Pressing the **Shift** key additionally moves the lower boundary, the **Control** key the upper boundary.

The vertical scale of the histogram is set using the scroll bar. The units are percents of the maximum grey value distribution. This setting has no influence on the function.

Parameters:

Input	Input image sequence
Output	Output image sequence
Channel	Selection of the channel numbers for the **Output** image after contrast enhancement
Clip Grey Values	Clipping of grey values to the **Low (L)** and **High (H)** output grey values boundaries
Input L	Lower boundary of grey value range **Input**
Input C	Center of grey value range **Input**
Input H	Upper boundary of grey value range **Input**
Output L	Lower boundary of grey value range **Output**
Output C	Center of grey value range **Output**
Output H	Upper boundary of grey value range **Output**

Contrast - Automatic

This function scales the grey values of an image sequence to the maximum possible range.

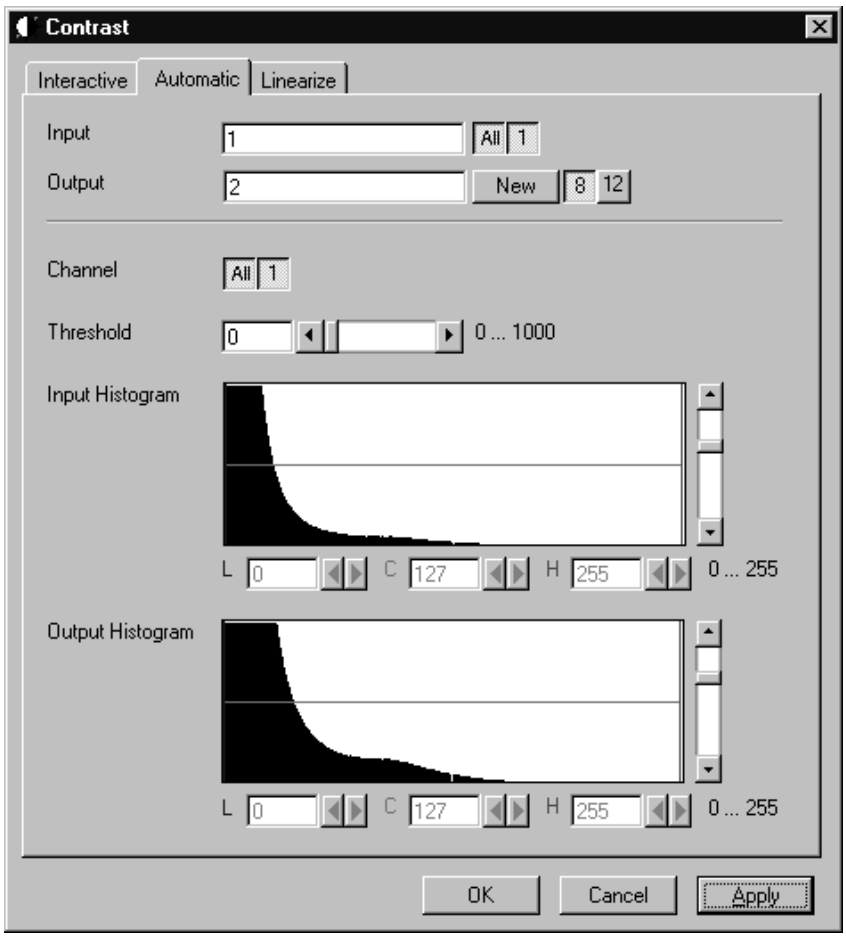


Fig. 5-14

The **Automatic** tab sheet of the **Contrast** dialog window must be selected.

This function enhances the contrast of an image sequence by spreading the grey value distribution over the maximum possible range. This function is used to achieve a better view of an image.

The light and dark grey value ranges with a low share of pixels are excluded from the operation by the parameter **Threshold**. The **Threshold** units are in thousandths of the total number of voxels. Using a value of 10 means that the scale interval is set so that 5/1000 of the total number of voxels on the light side, and 5/1000 of the total number of voxels on the dark side of the grey value distribution are excluded.

Input indicates the sequence to enhance. If it is a multichannel sequence, a single channel, all channels, or any number can be selected. The **Input** histogram shows the grey value distribution of the selected channels of the **Input** image sequence.

Output defines the name of the result sequence. It will get only those channels which are chosen by the **Input** parameter. The buttons labeled with 8 and 12 define the grey value (intensity) resolution in bit. Normally the result will get the same resolution as the **Input** sequence. A change will be needed if image sequences with different resolutions should be combined. Rising the grey value range to 12 bit will not enhance the display quality or measurement accuracy. The smooth and morphology functions will produce results with finer gradations.

The **Output** histogram shows the resulting histogram. They are not editable. The horizontal axis represents the grey values from 0 to the maximum, which is either 255 or 4095, depending whether the input is 8 bit or 12 bit. The vertical axis represents the pixel count. The vertical scale of the histogram is set using the scroll bar. The units are percentages of the grey value distribution maximum. This setting has no influence on the function.

Parameters:

Input	Input image sequence
Output	Output image sequence
Threshold	Exclusion value - 0...1000
Input L	Lower boundary of grey value range Input
Input C	Center of grey value range **Input**
Input H	Upper boundary of grey value range **Input**
Output L	Lower boundary of grey value range **Output**
Output C	Center of grey value range **Output**
Output H	Upper boundary of grey value range **Output**

Contrast – Linearize

This function scales a range of grey values of an image sequence to equal area fractions in the histogram.

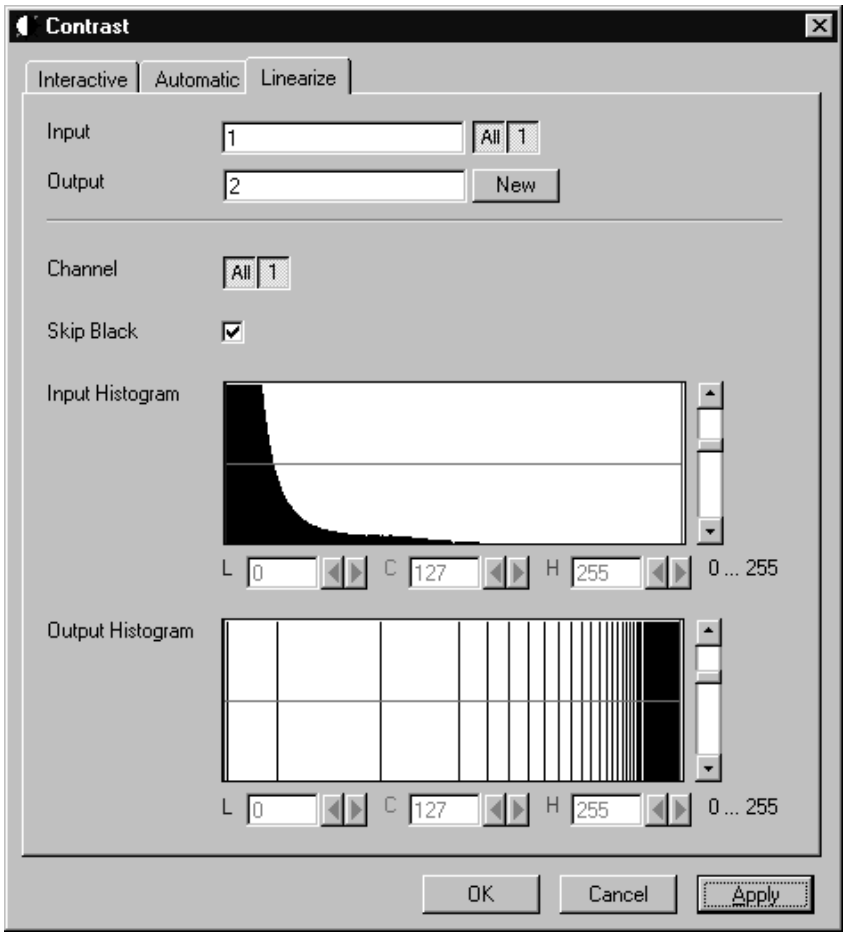


Fig. 5-15

The **Linearize** tab sheet of the **Contrast** dialog window must be selected.

This function enhances the contrast by linearizing the histogram of the image sequence to equal area fractions in the histogram. The areas (voxel count multiplied by grey value range) of all grey values in the **Output** histogram are the same. This function is used to achieve a better view of an image sequence. When **Skip Black** is checked the grey value 0 will not be taken into account for linearization.

Input indicates the sequence to enhance. If it is a multichannel sequence, a single channel, all channels, or any number can be selected. The **Input** histogram shows the grey value distribution of the selected channels of the **Input** image sequence.

Output defines the range of the result sequence. It will get only these channels which are chosen by the **Input** parameter. The grey value (intensity) resolution will be the same as the one from **Input**.

The **Output** histogram shows the resulting histogram. The horizontal axis represents the grey values from 0 to 255. The vertical axis represents the pixel count. The vertical scale of the histogram is set using the scroll bar. The units are percentages of the grey value distribution maximum. This setting has no influence to the function.

Parameters:

Image	Input image sequence
Output	Output image sequence
SkipBlack	0 - Grey value black is ignored 1 - Grey value black is taken into account
Input L	Lower boundary of grey value range Input
Input C	Center of grey value range Input
Input H	Upper boundary of grey value range Input
Output L	Lower boundary of grey value range Output
Output C	Center of grey value range Output
Output H	Upper boundary of grey value range Output

Smooth (Gauss)

This function performs a Gauss filter.

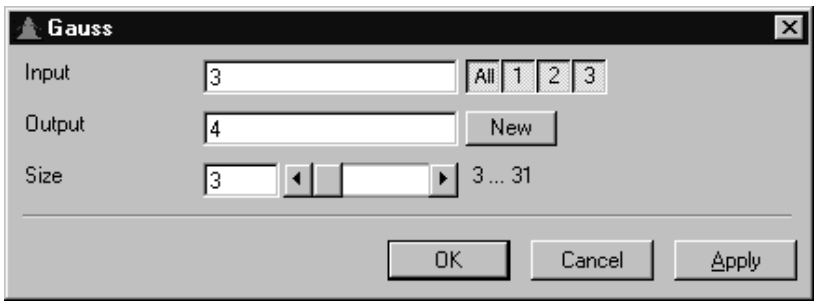


Fig. 5-16

The noise in the image sequence is reduced, the edge shape is nearly unchanged, local maxima are leveled, the dynamic range is reduced.

Image sequences should be smoothed before they are reconstructed or segmented. For most sequences a **Size** value of 3 is sufficient enough. If **Input** is a multichannel sequence, any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The grey value of every pixel is substituted by a weighted average of its surrounding neighbors. The neighbors are defined by a cube. The affected pixel is the central pixel of the filter cube. The weighted filter cube is approximated by a binomial distribution. The size of the filter cube is set using the **Size** scroll bar. Even numbers are set to the next odd value. The **Size** defines the strength of the smoothing.

Parameters:

Input	Input image sequence
Output	Output image sequence
Size	Filter size (3...31, only odd numbers)

Morphology

The following four functions perform basic operations of mathematical morphology on image sequences.

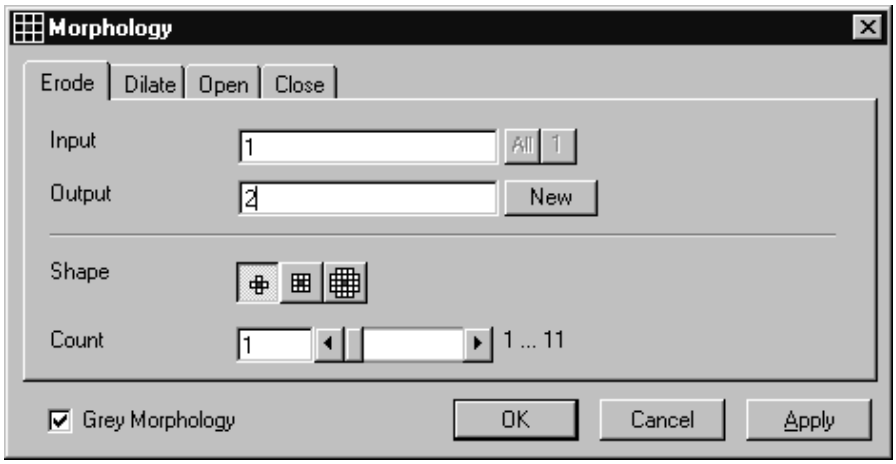


Fig. 5-17

As generalization of the morphology of two-dimensional images to three dimensions the structural elements are small volumina.

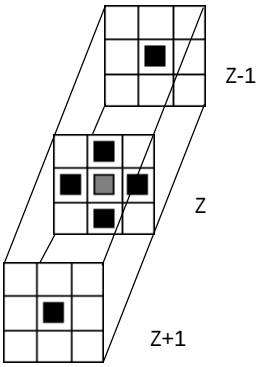
Literature

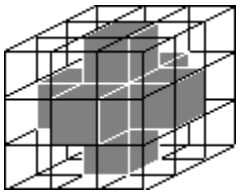
Bomans, M.; Höhne, K.-H.; Tiede, U.; Riemer, M.:
3D-Segmentation of MR Images of the Head for 3-D Display
IEEE Transactions on Medical Imaging 9, 1990, 177-183
Schiemann, T.; Bomans, M.; Tiede, U.; Höhne, K.-H.:
Interactive 3D-Segmentation of Tomographic Image Volumes
14. DAGM-Symposium Mustererkennung, Springer-Verlag 1992, 73-80

The input image sequence is analyzed voxel by voxel with a selected shape (**Shape**). The voxel to be analyzed is always the central voxel of the shape. The shape type determines which neighboring voxels are used to compute the resulting voxel.

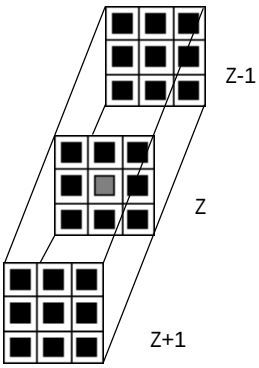
The following structural elements are available for all morphological operations. They represent approximated spheres with an increasing radius.

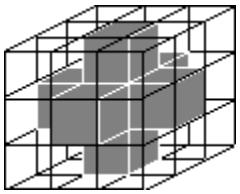
Sequential image: Volume view: Cross shape



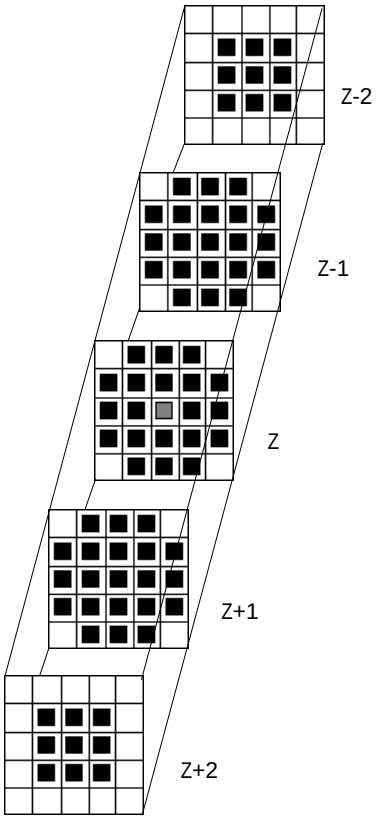


Sequential image: Volume view: Cross shape

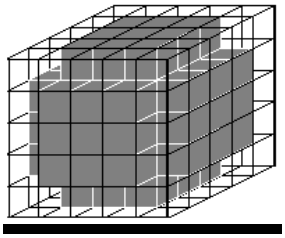




Sequential image:



Volume view:



Cube cross shape: created through application of "cube" and "cross" one after the other.

For regions (voxels) that are at the edge of the image sequence, it assumed for erosion that there are white voxels with a grey value of 255 (4095) outside the edge. For dilation, it is assumed that there are black voxels with the grey value 0 outside the image sequence.

If the **Grey Morphology** tickbox is activated, erosion sets the grey value of the central voxel to the minimum of all neighboring voxels affected by the structural element; dilation sets the grey value of the central voxel to the maximum.

If the **Grey Morphology** tickbox is not activated, the neighboring voxels are only distinguished by grey value 0 and non-0. For erosion the central voxel is set to 0 if any of the neighbors is 0. It is set to 255 (4095) if any neighbor is not 0. For dilation the central voxel is set to 255 (4095) if any of the neighbors is not 0. It is set to 0 if all neighbors are 0.

Erosion reduces the size of bright regions, separates thin connections between them, and makes small regions disappear. Dilation, on the other hand, makes bright regions of the image grow in size, fills gaps, and smoothes small contour details.

The result of erosion and dilation is called opening. On the one hand, this maintains to some extent the original size of the regions while not losing the smoothing effect of erosion on the image. This name stands for the operation of reducing convex bulges in the contour of the region. Thin connections between regions are eliminated, broken borders between regions are connected, and small regions disappear.

The opposite operation (first dilation, then erosion) is called closing. Concave bulges in the contours of regions are filled in; connections are formed between adjacent regions.

The following example illustrates the operations "Open" and "Close" in two dimensions:

Open = Erosion + Dilation



Fig. 5-18

Close = Dilation + Erosion



Fig. 5-19

The "cube cross" shape was used for the operations shown.

Morphology - Erode

This function erodes structures in an image sequence.

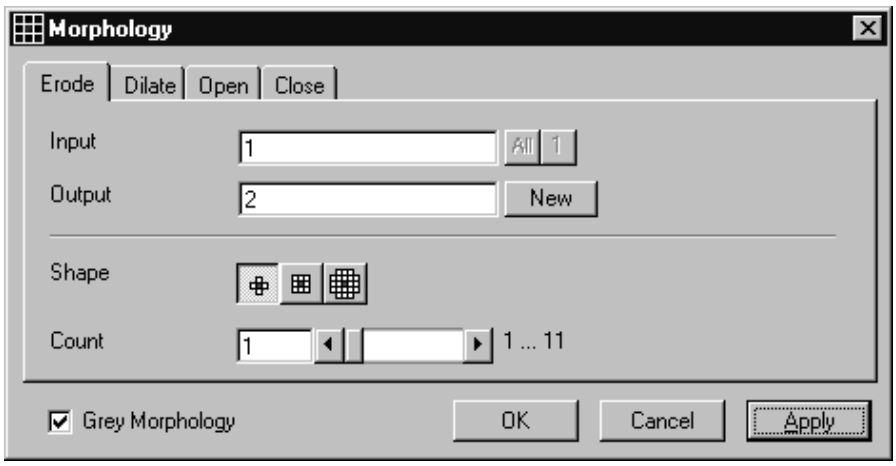


Fig. 5-20

In the **Morphology** dialog window, the tab sheet **Erode** must be selected.

Erosion makes bright regions smaller on a dark background. It also results in separation of thin connections between regions. Small regions disappear entirely.

If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results. The Input image sequence is eroded **Count** times with the shape **Shape**. The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:



If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Morphology - Dilate

This function dilates structures in an image sequence.

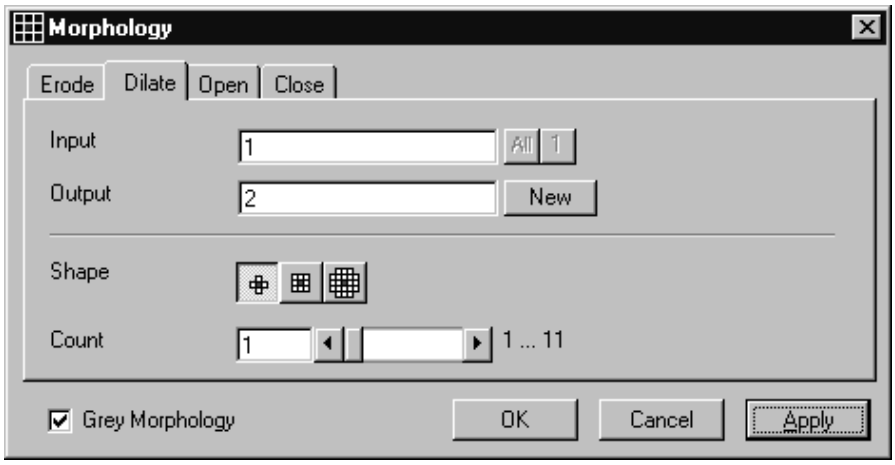


Fig. 5-21

In the **Morphology** dialog window, the tab sheet **Dilate** must be selected.

Dilation makes bright regions larger on a dark background. It also results in the filling of gaps and smoothing of small contour details.

If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The **Input** sequential image is dilated **Count** times with the shape **Shape**. The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:



If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Morphology - Open

This function carries out an opening.

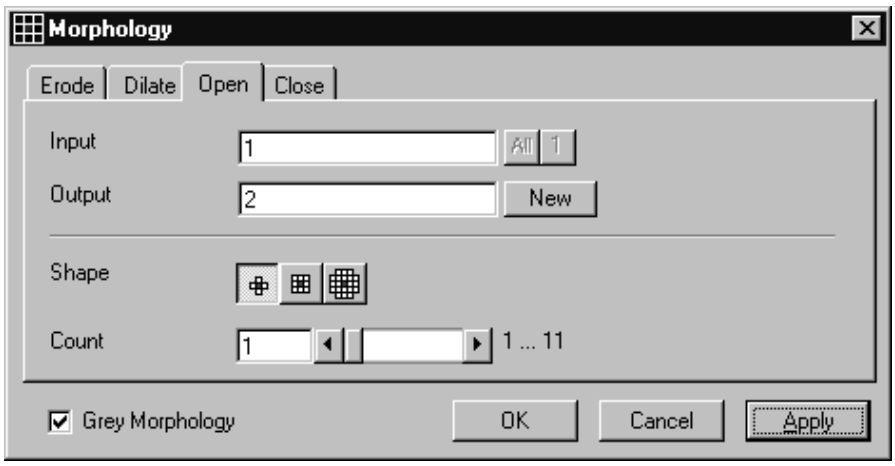


Fig. 5-22

In the **Morphology** dialog window, the tab sheet **Open** must be selected.

This function carries out an erosion followed by a dilation. For the most part, the opening maintains the original size of the regions. Thin connections between regions and small regions themselves disappear. Convex bulges in the contours of the regions are reduced. The opening is applied to the grey value image sequence **Input Count** times with the shape **Shape**. If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:



If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Morphology - Close

This function carries out a closing.

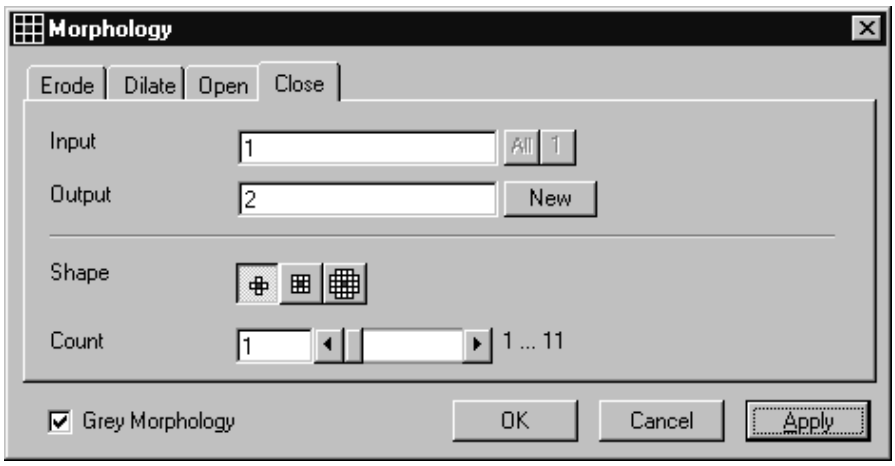


Fig. 5-23

In the **Morphology** dialog window, the tab sheet **Close** must be selected.

This function carries out a dilation followed by an erosion. For the most part, the closing maintains the original size of the regions. Connections are formed between adjacent regions; gaps and bright concave bulges in the contours of regions are filled in. The closing is applied **Count** times to the grey value image sequence Input with the shape **Shape**. If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:



If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Segment - Interactive

This function carries out a grey value segmentation by means of thresholding.

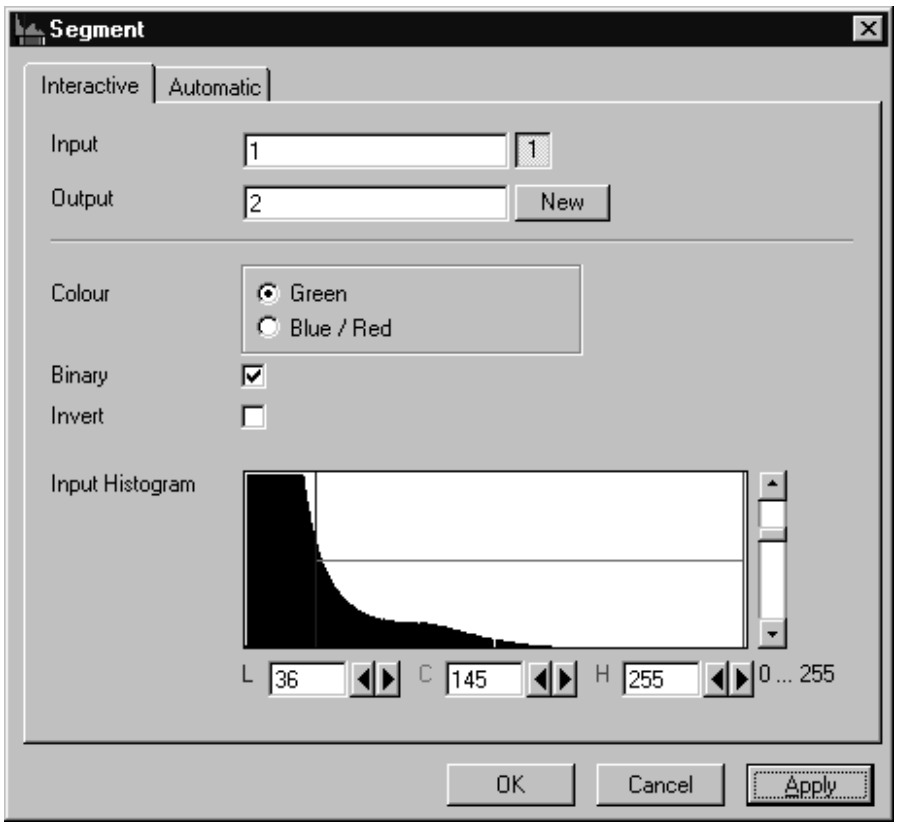


Fig. 5-24

The **Interactive** tab sheet of **Segment** dialog window must be selected.

Segmentation is especially used to generate binary regions. These are required for the measurement.

Two threshold values determine which grey value range of the **Input** image sequence is preserved and/or deleted in the **Output** image sequence. Only one channel of a multichannel sequence can be selected as **Input**. **Output** will always be a single channel sequence.

The vertical scaling of the histogram can be adjusted with the scroll bar at the right edge of the histogram. This setting has no influence on the function.

The thresholds **L**ow and **H**igh are determined either by moving the borderlines in the grey value histogram or by the scroll bars underneath. Furthermore, the values for **L**ow, **C**enter and **H**igh can be set through entry in the corresponding fields.

To move the lower (**L**) and upper (**H**) thresholds at the same time, move the vertical line in the grey value histogram or set the scroll bar (**C**).

The **Green** and **Blue/Red** option buttons of the parameter **Colour** determine whether the voxels within (**Green**) or outside (**Blue/Red**) of the grey value interval [**L, H**] are displayed with the corresponding colour.

If **Green** is selected, the voxels within the selected interval are highlighted in green. The rest of the image retains its original grey values. The voxels with the grey values **L**ow and **L**ow+1 are displayed in blue. The voxels with the grey values **H**igh and **H**igh-1 are displayed in red.

If **Blue/Red** is selected, the voxels with grey values within the interval **L**ow, **H**igh remain unchanged. Voxels with grey values less than **L**ow are highlighted in blue; those with grey values higher than **H**igh are highlighted in red.

If the **Invert** option is selected, the grey values outside the defined interval will be segmented.

If the option **Binary** is selected, then all grey values in the range from **L**ow to **H**igh will be set to white (grey value 255) in the **Output** image sequence, while all others will be set to black (grey value 0). If the option is not selected, the grey values within the selected interval remain unchanged, while those outside the range will be set to black. The measurement function accepts both results without any difference in the results.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Colour	Green - Selected interval is displayed in green
Blue/Red	Grey values below the selected interval are displayed in blue, grey values above in red
Binary	0 - Selected voxels retain the original grey value 1 - Selected voxels are set to grey value 255, the rest to grey value 0
Invert	0 - Grey values inside the selected interval are segmented 1 - Grey values outside the selected interval are segmented
L	Low grey value threshold
C	Center of threshold interval
H	High grey value threshold

Segment - Automatic

The function carries out an automatic grey value segmentation by means of thresholding.

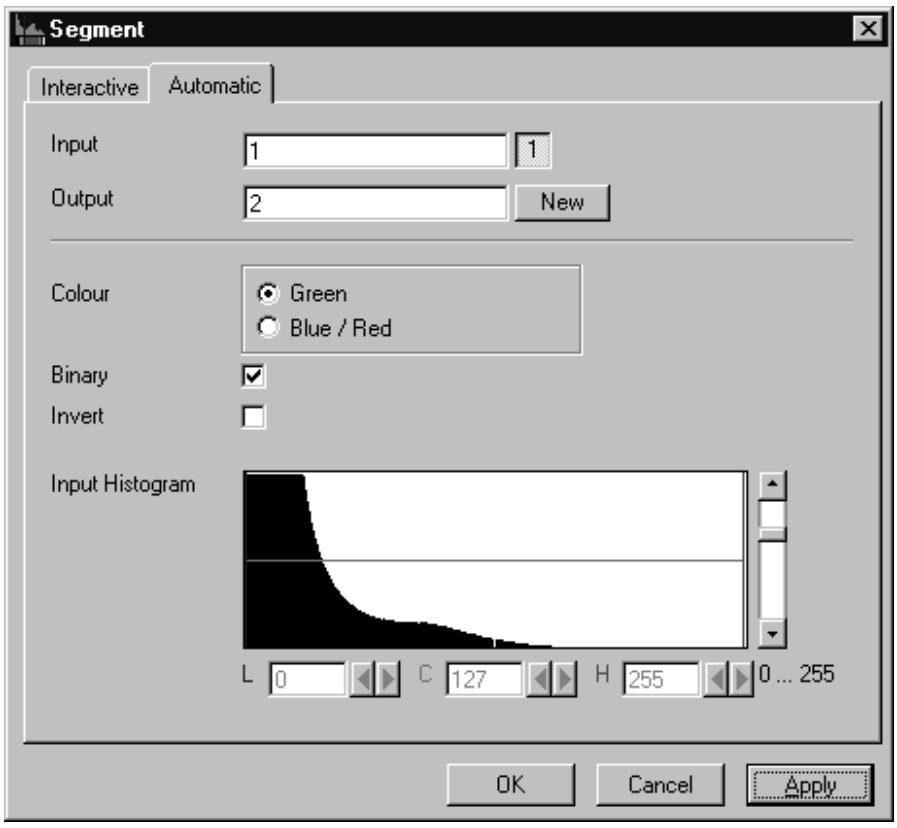


Fig. 5-25

The **Automatic** tab sheet of the **Segment** dialog window must be selected. Segmentation is especially used to generate binary regions. These are required for the measurement.

The function calculates the two strongest local minimums in the histogram of the **Input** image sequence. These values are used for the discrimination. Only one channel of a multichannel sequence can be selected as **Input**. **Output** will always be a single channel sequence. The vertical scaling of the histogram can be adjusted with the scroll bar at the right edge of the histogram. This setting has no influence on the function.

The **Green** and **Blue/Red** option buttons of the parameter **Colour** determine whether the voxels within (**Green**) or outside (**Blue/Red**) of the grey value interval [**L, H**] are displayed with the corresponding colour.

If **Green** is selected, the voxels within the selected interval are highlighted in green. The rest of the image retains its original grey values. The voxels with the grey values **L**ow and **L**ow+1 are displayed in blue. The voxels with the grey values **H**igh and **H**igh-1 are displayed in red.

If **Blue/Red** is selected, the voxels with grey values within the interval **L**ow, **H**igh remain unchanged. Voxels with grey values less than **L**ow are highlighted in blue; those with grey values higher than **H**igh are highlighted in red.

If the **Invert** option is selected, the grey values outside the defined interval will be segmented.

If the option **Binary** is selected, then all grey values in the range from **L**ow to **H**igh will be set to white (grey value 255 (4095)) in the **Output** image sequence, while all others will be set to black (grey value 0). If the option is not selected, the grey values within the selected interval remain unchanged, while those outside the range will be set to black.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Colour	Green - Selected interval is displayed in green Blue/Red - Grey values below the selected interval are displayed in blue, grey values above in red
Binary	0 - Selected voxels retain the original grey value 1 - Selected voxels are set to grey value 255 (4095), the rest to grey value 0
Invert	0 - Grey values inside the selected interval are segmented 1 - Grey values outside the selected interval are segmented
L	Low grey value threshold
C	Center of threshold interval
H	High grey value threshold

Boolean - And

This function carries out a bit-by-bit **And** calculation for the image sequences **Input 1** and **Input 2**.

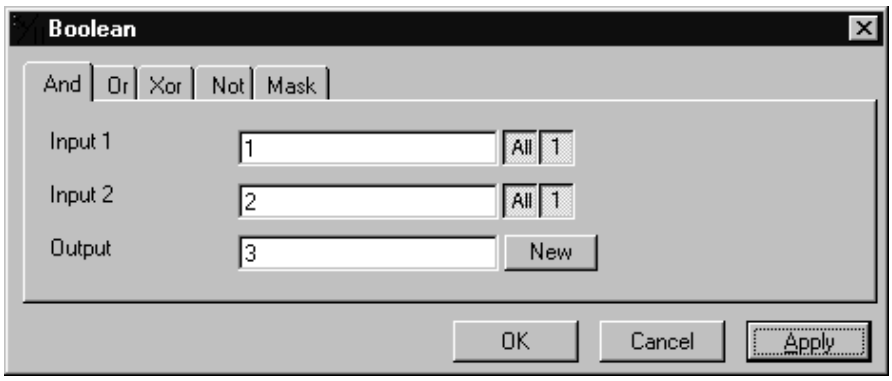


Fig. 5-26

The **And** tab sheet of the **Boolean** dialog window must be selected.

This function is especially well suited for masking images.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Boolean - Or

This function carries out a bit-by-bit **Or** calculation for the images **Input 1** and **Input 2**.

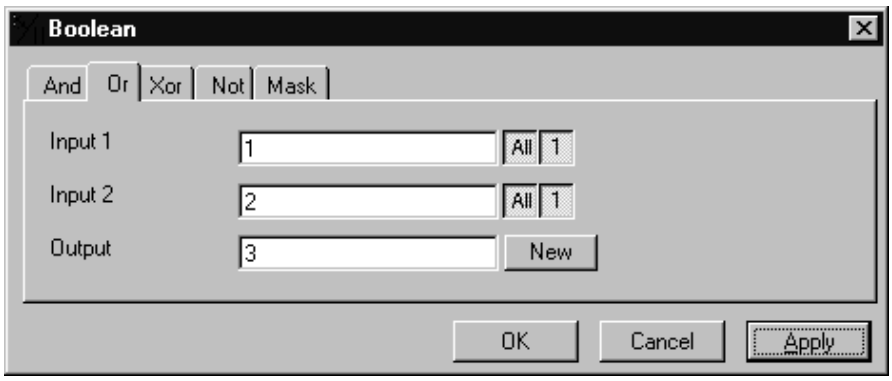


Fig. 5-27

The **Or** tab sheet of the **Boolean** dialog window must be selected.

This function can be used to combine binary masks or regions.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Boolean - Xor

This function carries out a bit-by-bit **Xor** calculation for the images **Input 1** and **Input 2**.

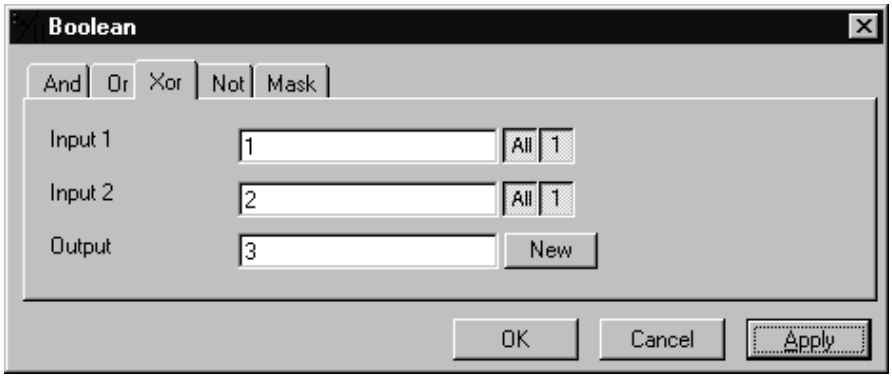


Fig. 5-28

The **Xor** option button of the **Function** option group in the **Boolean** dialog window must be selected.

This function can be used to combine binary masks or regions.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Boolean - Not

This function carries out a bit-by-bit negation of an image.

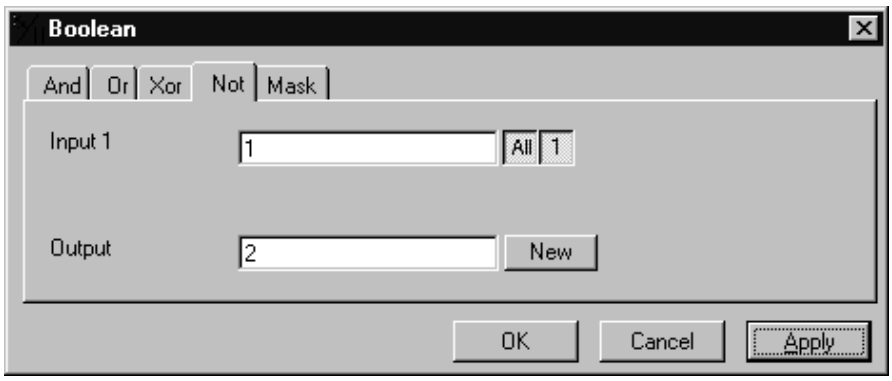


Fig. 5-29

The **Not** tab sheet of the **Boolean** dialog window must be selected.

If **Input** is a multichannel sequence any number or combination can be selected.

Parameters:

Input	Input image sequence
Output	Resulting image sequence

Boolean - Mask

This function masks a grey value image sequence.

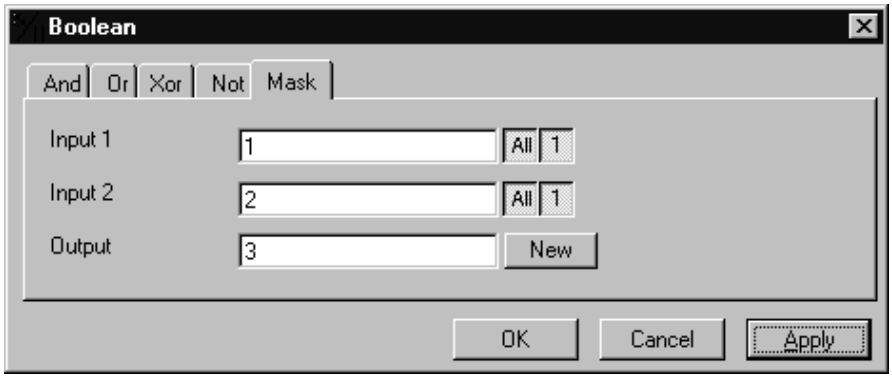


Fig. 5-30

The **Mask** tab sheet of the **Boolean** dialog window must be selected.

This function modifies the **Output** image sequence depending on the mask image sequence used.

If the grey value in **Input 2** is higher than 0, then the voxel values are copied from **Input 1** to the image sequence **Output**. If the grey value of the voxel is 0, then the voxel value of the **Output** image sequence is taken over.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 2** must be 1 or the same as for **Input 2**. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Scrap

This function deletes or selects objects in a specified size range.

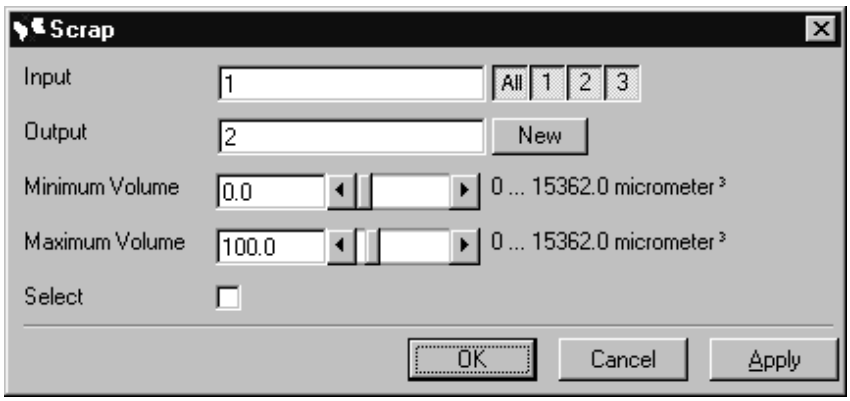


Fig. 5-31

The operation deletes or selects objects on the basis of their total volume in voxels. Objects with a volume within the range **MinVolume** to **MaxVolume** are effected.

To delete objects outside the range, the parameter **Select** must be active. If the parameter is not activated objects outside the defined volume range are deleted.

Parameters:

Input	Input image sequence
Output	Output image sequence
MinVolume	Minimum object size
MaxVolume	Maximum object size
Select	0 - Select the objects outside the size range 1 - Select the regions within the size range

Fill Holes

This function fills holes in all objects.



Fig. 5-32

All holes in objects are filled by this operation. Holes are structures, which have a grey value of 0 and are surrounded completely by voxels with a grey value not equal to 0. It is assumed that regions outside the image are black. Holes, which touch the image border, are retained.

Parameters:

Input	Input image sequence
Output	Output image sequence

5.3.4 Functions in the View Menu

Render - Surface

This function displays an image sequence according to the **gradient shading method**.

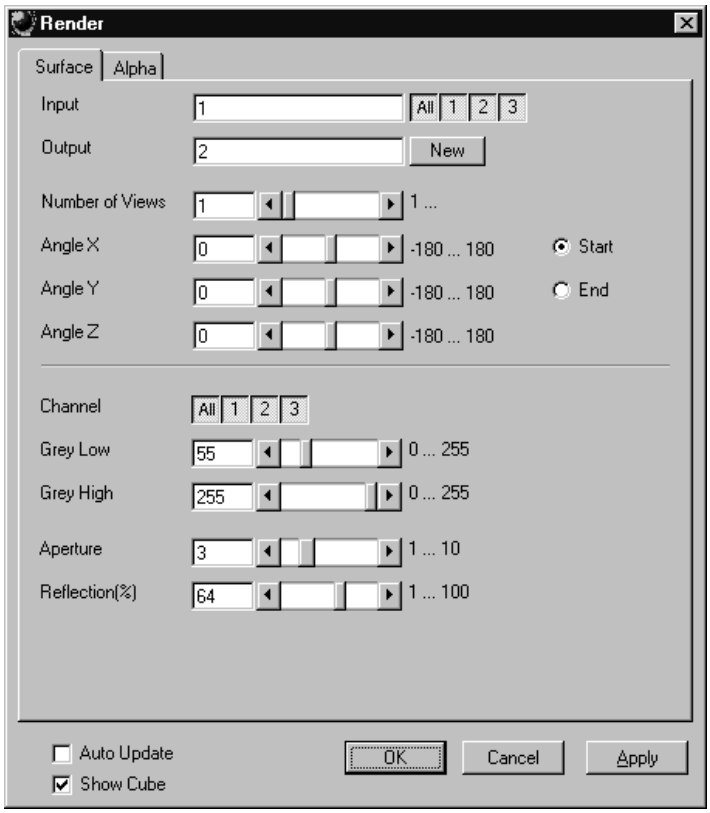


Fig. 5-33

The **Surface** tab sheet of the **Render** dialog window must be selected.

Method

The **Input** sequence defines the data to be reconstructed. If it is a multichannel sequence one or all channels can be selected for the reconstruction.

Output sets the name of the result image (sequence). If the sequence exists it is overwritten. Pressing the button **New** will generate a new name (number). The size of the sequential images in **Output** is determined by the size of the sequential images in **Input**.

Number of Views determines the number of reconstructions which should be computed. The radio buttons **Start** and **End** define which angle settings are currently shown. A definition for the angle **End** is only necessary if **Number of Views** is higher than 1. If this is true the result sequence will get views from the **Start** to the **End** angle definition. The other reconstructions are determined through the linearly interpolated intermediate angles. The direction of view is determined from the angles as follows:

The angle **Angle Z** determines the rotation of the direction of view on the Z-axis. The angle **Angle Y** determines the rotation of the direction of view on the Y-axis that has been rotated by the angle **Angle Z**. The angle **Angle X** determines the rotation of the direction of view on an X-axis that is rotated by **Angle Z** and **Angle Y**.

Channel defines if the following parameters are valid for **All** or just for one. Defining the thresholds for the channels independently is useful if the grey value boundaries of the objects differ too much in the different channels. The thresholds **Grey Low** and **Grey High** define the grey value range of the objects.

The parameter **Aperture** is a measure of the size of the highlights. Small values generate large highlights. Large values generate small highlights (similar to a spot).

Use the parameter **Reflection** to control the ratio of diffuse and reflective brightness components, i.e., the overall basic brightness compared with the highlights. When the value of **Reflection** is low, the highlights predominate; when the values are high, the region appears to be uniformly illuminated and the highlights are not so pronounced. When **Auto Update** is selected, the reconstruction is updated automatically whenever a parameter is modified (except **Input, Output**, or **Number of Views**). **Show Cube** defines whether a wire frame cube is shown in the **Display window** or not.

Application

This method can be applied, if the structures in the **Input** sequence can be segmented by grey value thresholding. Because the gradient is calculated for every pixel, the **Output** appears in very fine detail.

Noisy **Input** sequences must be smoothed (function **Smooth**) before rendering, otherwise the surface appears rough.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Number of Views	Number of reconstructions to be calculated
Angle X	Angle of rotation on the X-axis, start position
Angle Y	Angle of rotation on the Y-axis, start position
Angle Z	Angle of rotation on the Z-axis, start position
Channel	All - The following parameters are valid for all channels X - The following parameters are valid for the selected channel only
Grey Low	Low grey value threshold of the region to be displayed
Grey High	High grey value threshold of the region to be displayed
Aperture	Measure of the extent of the highlights
Reflection	Weight of the defuse brightness components in comparison to the highlights
Auto Update	0 - Function execution is performed on **OK** or **Apply** 1 - Function execution for the current angle is performed on any parameter change
Show Cube	0 - The wire frame cube is not shown 1 - The wire frame cube is shown in the **Display window**

Render - Surface: Method Description

This method displays the surface of structures in the **Input** sequence shaded as if a light illuminated it. The position of the light is behind the view point with parallel rays in the direction of the sequence.

The input sequence is segmented into object and background by grey value thresholding: object voxels are within the grey value range **Grey Low** to **Grey High**.

Each **Output** pixel corresponds to a point at the surface at which the ray in view direction through the **Output** pixels hits the surface. All rays are parallel.

The surface normal required for shading in this gradient renderer is the grey value gradient in the **Input** volume at the surface voxel position. It is not the geometric surface normal. The grey value gradient is determined from the grey values in a 3x3x3 cube around the surface voxel by averaging e.g. the x-gradient in y- and z-direction [4].

There is no depth cueing (far objects would appear darker).

The illumination model is a Phong model [1] (surface normal is determined for each **Output** pixel) with diffuse reflection and specular reflection. Diffuse reflection means that the surface reflects light with equal intensity in all directions. The brightness of a given surface patch depends not on the view-direction, but only on the angle between light and surface normal. Specular reflection is observed on shiny surfaces as a highlight. The light is reflected as from a mirror. The maximum intensity is observed when the view direction is the one of the mirrored light direction.

Render - Alpha

This function displays an image sequence according to the **alpha rendering method**.

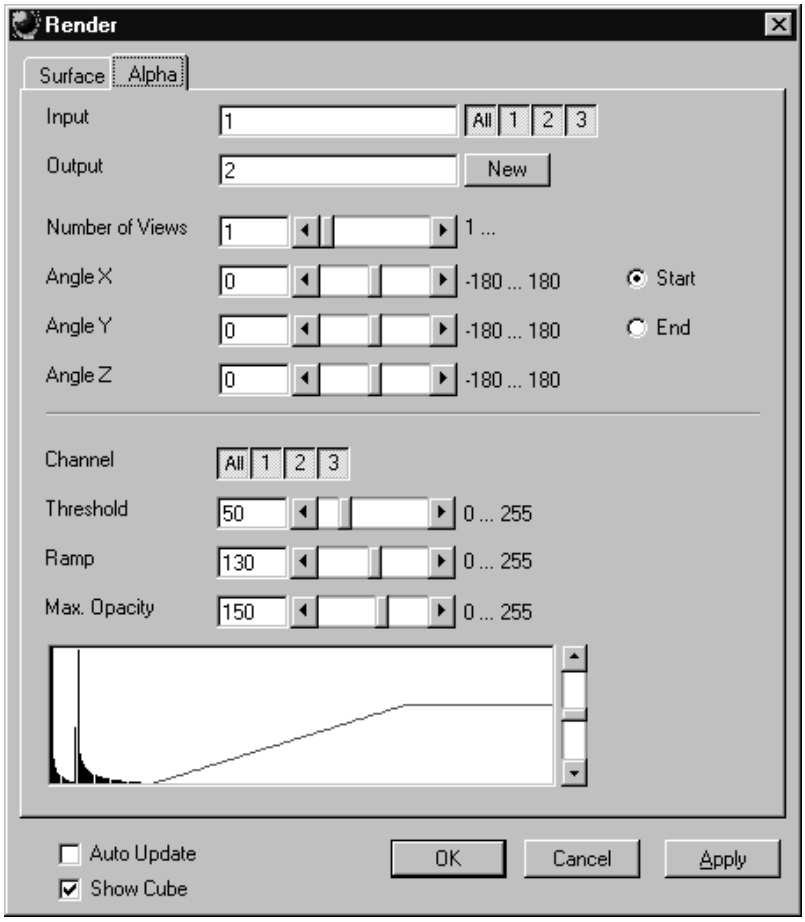


Fig. 5-34

The **Alpha** tab sheet of the **Render** dialog window must be selected.

One or more reconstructions of the input image sequence are computed according to the alpha rendering method. This type of reconstruction should be used if there is no possibility to segment the structures in the image sequence and also if the objective is to make deeply layered structures visible.

Method

The **Input** sequence defines the data to be reconstructed. If it is a multichannel sequence one or all channels can be selected for the reconstruction.

Output sets the name of the result image (sequence). If the sequence exists it is overwritten. Pressing the button **New** will generate a new name (number). The size of the sequential images in **Output** is determined by the size of the sequential images in **Input**.

Number of Views determines the number of reconstructions which should be computed. The radio buttons **Start** and **End** define which angle settings are currently shown. A definition for the angle **End** is only necessary if **Number of Views** is higher than 1. If this is true the result sequence will get views from the **Start** to the **End** angle definition. The other reconstructions are determined through the linearly interpolated intermediate angles.

The direction of view is determined from the angles as follows:

The angle **Angle Z** determines the rotation of the direction of view on the Z-axis. The angle **Angle Y** determines the rotation of the direction of view on the Y-axis that has been rotated by the angle **Angle Z**. The angle **Angle X** determines the rotation of the direction of view on an X-axis that is rotated by **Angle Z** and **Angle Y**.

Channel defines if the following parameters are valid for **All** or just for one. Defining the opacity for the channels independently is useful when the brightness and contrast of the channels differ too much. **Threshold** defines the range with no opacity. It is completely transparent. The range starts at grey value 0.

The length of slope is defined by **Ramp**. The maximum opacity value is set with the parameter **Max. Opacity**. This range ends at the maximum grey value. The **Opacity Table** shows the grey value histogram of Input with the opacity definition as a red line.

When **Auto Update** is selected, the reconstruction is updated automatically whenever a parameter is modified (except **Input, Output**, or **Number of Views**). **Show Cube** defines whether a wire frame cube is shown in the **Display window** or not.

Application

1. This method can be applied, if the structures in the Input sequence are unsharp so that objects are poorly defined by their grey value.
2. In this case, the Opacity Table is defined as a ramp. Low grey values have weight 0 to suppress the background voxels. The opacity rises with increasing grey values, depending on the parameter Ramp. The value of Max. Opacity defines the weight of the high grey values. High grey values above a threshold have weight 255 to show the "object" voxels unsuppressed. Of course a smooth step can be used.
3. The result is a display with inside structures shining through. A 3D impression can be obtained by rendering with several view directions.
4. In contrast to this, a voxel renderer like the gradient renderer would display only the surface of objects that are defined by grey value-thresholds. This surface would appear shaded as if illuminated by a light.
5. The method can also be applied to visualize pronounced structures within other enclosing structures, if the structures have different grey value ranges.
6. In this case, the Opacity Table is defined as a step. Low grey values (background) have weight 0. High grey values (inside structures) have maximum weight.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Number of Views	Number of reconstructions to be calculated
Angle X	Angle of rotation on the X-axis, start position
Angle Y	Angle of rotation on the Y-axis, start position
Angle Z	Angle of rotation on the Z-axis, start position
Channel	All - The following parameters are valid for all channels X - The following parameters are valid for the selected channel only
Threshold	Grey value where the opacity starts rising
Ramp	Length of the opacity slope
Max. Opacity	Maximum opacity value
Opacity Table	Maximum opacity value
Auto Update	0 - Function execution is performed on OK or Apply 1 - Function execution is performed on any parameter change
Show Cube	0 - The wire frame cube is not shown 1 - The wire frame cube is shown in the Display window

Render - Alpha: Method Description

Each **Output** pixel is a weighted sum of the **Input** voxels along a ray in view direction through the **Input** sequence. Each **Input** voxel has an opacity value, dependent only on its grey value. The opacity values are defined by the parameters **Threshold**, **Ramp**, and **Max. Opacity**.

Accumulation of pixels proceeds along the ray from back to front, i.e. from far pixels to near pixels. If a new pixel is added, it increases the result intensity by its grey value weighted by the opacity value, and attenuates the previously accumulated intensity according to the opacity value. Full intensity stops accumulation.

This calculation must be repeated for each pixel of the ray to generate one **Output** pixel. Then for each **Output** pixel to produce a 2D **Output** image for the selected view-angle. Then for each view-angle to produce an output sequence for **Number of Views** different view angles.

Render - References

[1] J.D. Foley,A.van Dam, S. K. Feiner, J.F.Hughes, Computer Graphics: Principles and Practice, Addison Wesley, Reading, MA, 1990.

[2] M. Levoy, Display of Surfaces from Volume Data, IEEE Computer Graphics & Applications, May 1988, 29-37.

[3] J. Ylä-Jääski, F.Klein, O. Kübler, Fast Direct Display of Volume Data for Medical Diagnosis, VGIP:Graphical Models and Image Processing 53,1991,7-18.

[4] K.H. Höhne, R. Bernstein, Shading 3D-Images from CT Using Gray-Level Gradients, IEEE Transactions on Medical Imaging, 5, 1986, 45-47.

[5] D.Gordon, R.A. Reynolds, Image Space Shading of 3-Dimensional Objects, CVGIP 29, 1985, 361-376.

5.3.5 Functions in the Measurement Menu

Measurement Concept

Measurement is based on regions (objects) in three-dimensional space. Segmenting an image sequence generates these. The image segmentation process produces a mask image that defines the region.

A region is a group of voxels that touch at the surfaces or at the edges, but not at the corners (18 voxel neighborhood).

This is illustrated by the following example. The voxels marked black in sequential image Z-1, Z, Z+1 all belong to the same region as the grey central voxel in sequential image Z. The volume view shows the neighborhood interrelationships as a 3D projection.

Sequential image: Volume view:

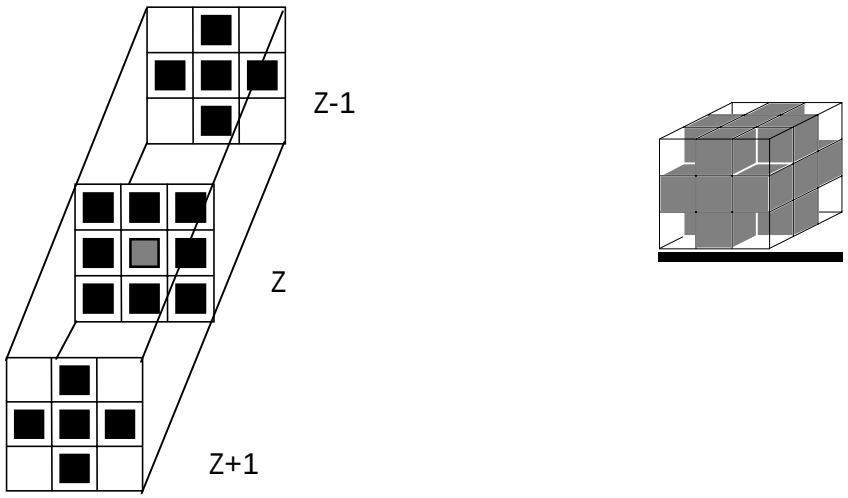


Fig. 5-35

Measurement Process

The measurement process consists of three steps: region definition, checking of the validity of the regions, and feature calculation.

Region definition:	- Automatically from the mask image
Region validation check depends on:	- Minimum volume - Measurement condition
Feature calculation depends on	- Shape of the region - Densitometric value distribution of the region - Feature parameters

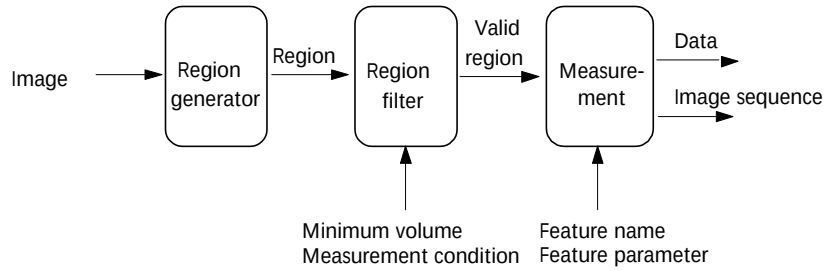


Fig. 5-36

All regions found are checked according to certain conditions. The voxel volume of each region must be equal to or greater than **MinVolume**. The measurement condition must be fulfilled. Only those regions that meet all the conditions are valid for the measurement. The region can be measured or labeled. Measurement is a process that produces data. Labeling is a process that generates an image volume.

Automatic Object Measurement – Object Features

A measurement feature describes a region characterized by a number (e.g. volume, area or a densitometrical statistic). The features can be selected on the **Object Features** and **Volume Features** tab sheets.

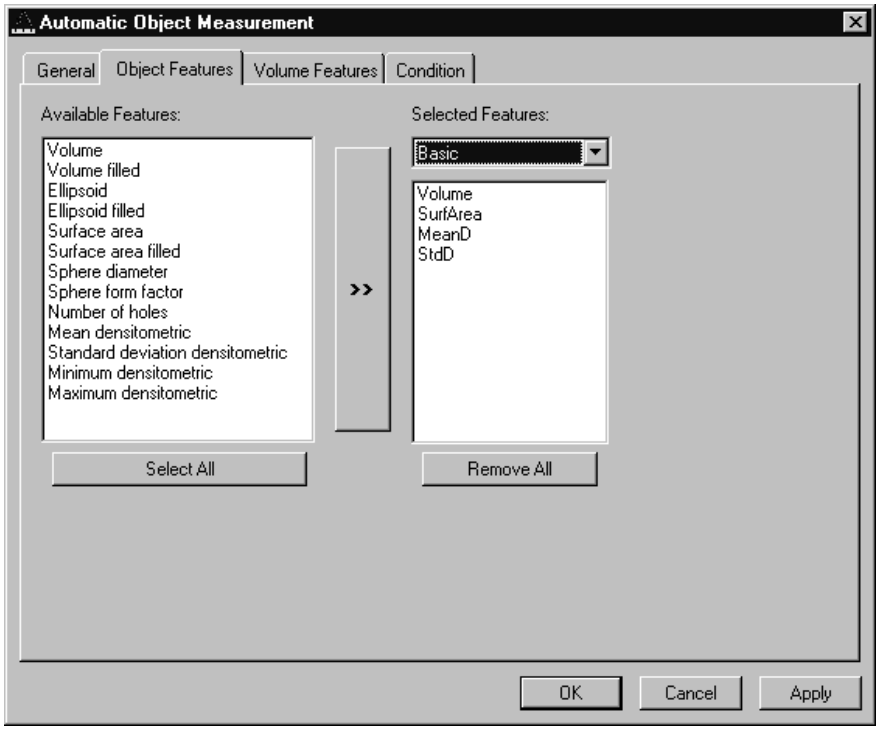


Fig. 5-37

The scalings and units are taken automatically from the assigned sequence.

The measurement features can be selected individually for each measurement. The object features generate a result value for every single object.

The dialog shows two lists. One shows the **Available Features** as groups (on the left). The other one shows the **Selected Features**. Double-clicking on items of the left list will add the **Selected Features** to the right list. Double-clicking on an item of the right list will remove this item from the list. **Selected Features** can also be transferred by clicking on the button in the middle (<< / >>) of the dialog.

The combo box above the right list represents predefined feature lists. Selecting one of the entries will fill the right list with these features; previously selected features will be overwritten.

The button **Select All** will copy all features to the list of selected features.

The button **Remove All** will clear the list of selected features.

Clicking on the **Apply** button will execute the measurement process and switch to the **General** tab sheet of the dialog.

Parameters:

Available Features	List of available object features
Selected Features	List of selected object features
Select All	Select all available object features for measurement
Remove All	Remove all object features from the selected features list

The following sections describe all measurement features which are defined in the system.

Object Features (geometric)

If **Object Features** are selected, one set of measurement data is calculated for each object.

Group Name	Name	Description
Volume	Volume	Volume of the object.
Volume Filled	VolumeF	Volume of the filled object.
Ellipsoid	EllipseA	Length of the main axis of the ellipsoid with the same geometrical moment of inertia as the object.
	EllipseB	Length of the middle axis of the ellipsoid with the same geometrical moment of inertia as the object.
	EllipseC	Length of the minor axis of the ellipsoid with the same geometrical moment of inertia as the object.
Ellipsoid filled	EllipseAF	Length of the main axis of the ellipse with the same geometric moment of inertia as the filled object.
	EllipseBF	Length of the middle axis of the ellipse with the same geometric moment of inertia as the filled object.
	EllipseCF	Length of the minor axis of the ellipse with the same geometric moment of inertia as the filled object.
Surface Area	SurfArea	Surface area of the object.
Surface Area Filled	SurfAreaF	Surface area of the filled object.
Sphere Diameter	Dsphere	Diameter of the sphere with the same volume. $\sqrt{6 * VOLUMEF / \pi}$
Sphere Form Factor	Fsphere	Form factor of the object. $6 \cdot \sqrt{\pi} \cdot \frac{VOLUMEF}{\sqrt{SURFAREAF^3}}$
Number of Holes	Nparts	Number of holes within an object.

Object Features (densitometric)

Group Name	Name	Description
Mean Densitometric	MeanD	Densitometric mean value of an object.
Standard Deviation Densitometric	StdD	Standard deviation of the densitometric values of an object.
Minimum Densitometric	MinD	Minimum grey value of an object.
Maximum Densitometric	MaxD	Maximum grey value of an object.

Automatic Object Measurement - Volume Features

A measurement feature describes a region characterized by a number (e.g. volume, area, or a densitometrical statistic). The features can be selected on the **Object Features** and **Volume Features** tab sheets.

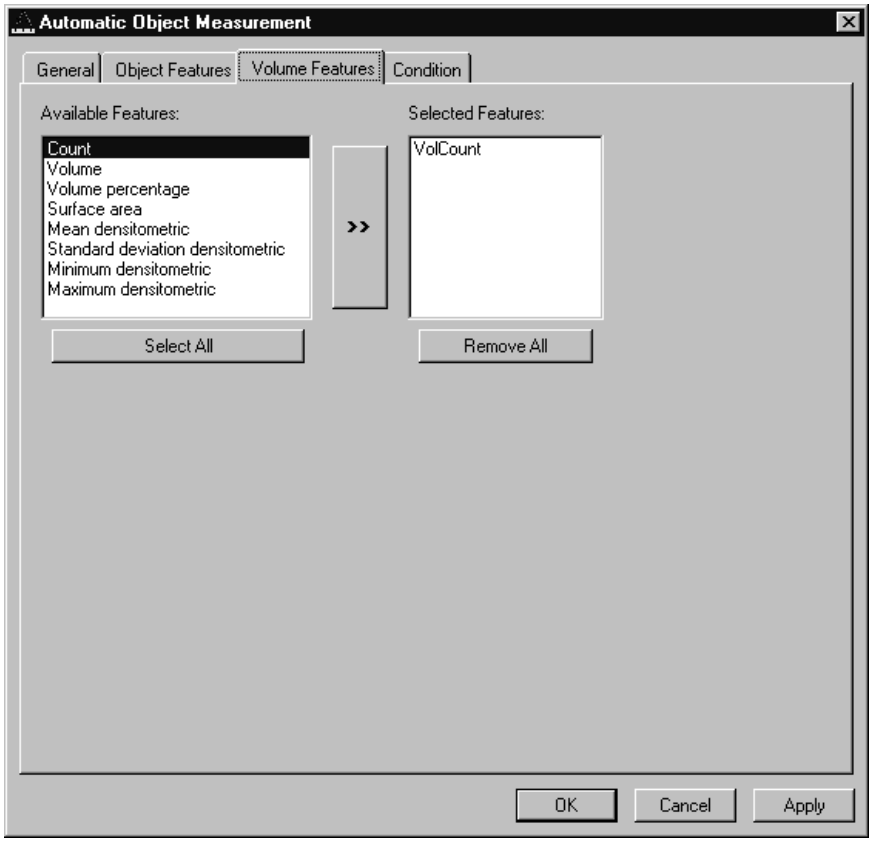


Fig. 5-38

The measurement features can be selected individually for each measurement. The object features generate a result value for every single object.

The dialog shows two lists. One shows the **Available Features** as groups (on the left). The other one shows the **Selected Features**. Double-clicking on items of the left list will add the **Selected Features** to the right list. Double-clicking on an item of the right list will remove this item from the list. **Selected Features** can also be transferred by clicking on the button in the middle (<< / >>) of the dialog.

The combo box above the right list represents predefined feature lists. Selecting one of the entries will fill the right list with these features; previously selected features will be overwritten.

The button **Select All** will copy all features to the list of selected features.

The button **Remove All** will clear the list of selected features.

Clicking on the **Apply** button will execute the measurement process and switch to the **General** tab sheet of the dialog.

Parameters:

Available Features	List of available object features
Selected Features	List of selected object features
Select All	Select all available object features for measurement
Remove All	Remove all object features from the selected features list

Volume Features (geometric)

The volume-related measurement generates one measured value per image sequence. The following table contains the predefined volume characteristics.

Group Name	Name	Description
Count	VolCount	Number of regions measured.
Volume	VolVolume	Total volume of all regions.
Volume Percentage	VolVolumeP	Total volume of all regions, in relation to the volume of the image sequence.

Volume Features (densitometric)

Group Name	Name	Description
Surface Area	VolSurfArea	Total surface area of all regions.
Mean Densitometric	VolMeanD	Mean grey value of all regions.
Standard Deviation Densitometric	VolStdD	Grey value standard deviation of all regions.
Minimum Densitometric	VolMinD	Minimum grey value in the image sequence.
Maximum Densitometric	VolMaxD	Maximum grey value in the image sequence.

Automatic Object Measurement - Condition

The measurement conditions are used to limit the objects to be evaluated (e.g. only objects with defined minimum value). All objects are tested against the defined conditions. If the conditions are fulfilled the feature values are written to the data table.

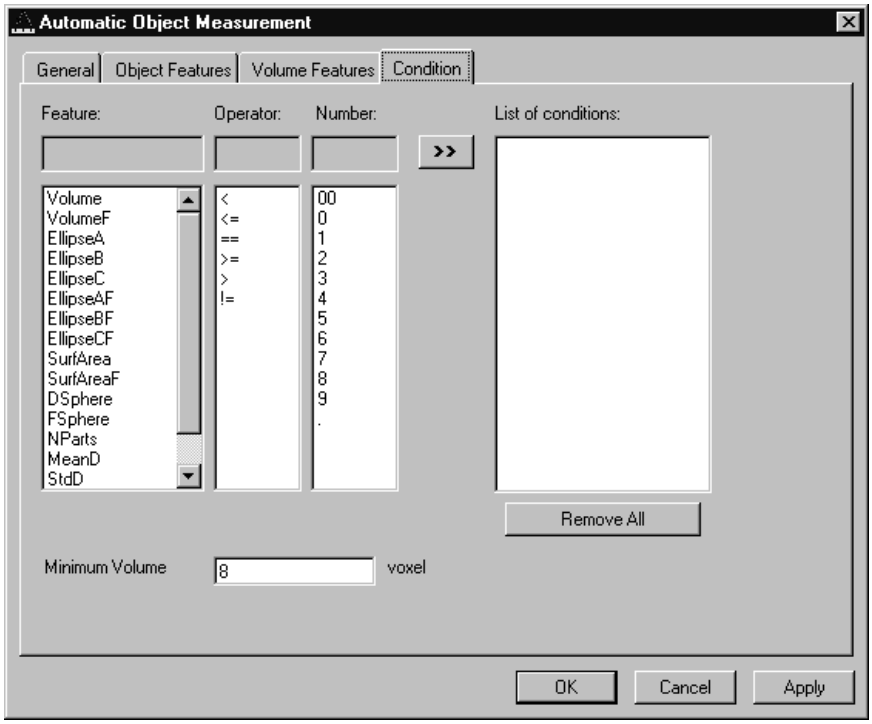


Fig. 5-39

To define the following parameter select the **Condition** tab sheet of the **Automatic Object Measurement** dialog window.

The list on the very left at the dialog shows all the measurement **Features**. The second list provides the comparison **Operators** and the next **Numbers** to define a value. This gives the possibility to compose an expression to test a feature value against a constant value. The fields above the lists will show the composed (selected) string. Clicking on the desired list entry does the selection. The button with the „>>„ characters adds this string to the **List of Conditions**. All lines of the **List of conditions** are combined with the AND expression automatically. To remove a condition line double-click on it.

The parameter **Minimum Volume** defines the minimum voxel volume for the measurement. This is an easy way to eliminate very small regions caused by noisy sequences and segmentation process.

The button **Remove All** will clear the list of defined conditions.

Clicking on the **Apply** button will execute the measurement process and switch to the **General** tab sheet of the dialog.

Parameters:

Feature	List of available object features
Operator	List of available condition operators
Number	List of numbers to compose the value
List of conditions	Defined condition list
Remove All	Remove all entries from the **List of conditions**
Minimum Volume	Minimum object volume in voxel

Automatic Object Measurement - General

This function carries out an automatic measurement and labeling.

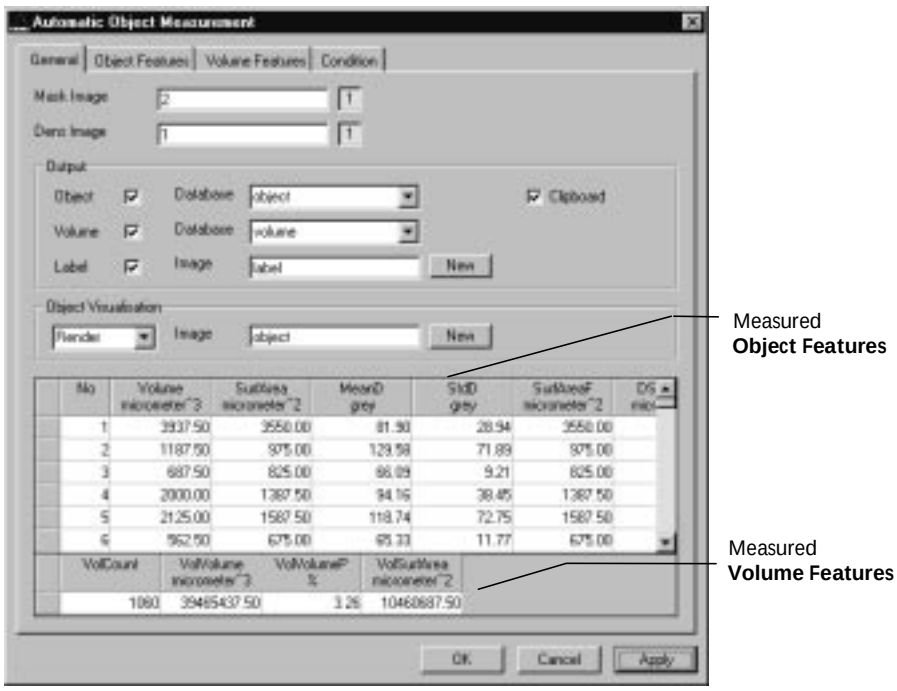


Fig. 5-40

The regions must be defined by an image sequence **Mask Image** (the objects must be separated from one another by black voxels with the grey value 0). This sequence is generated with the function **Segment**. If it is a multichannel sequence a single channel has to be chosen.

The image **Dens Image** is needed for the measurement of the densitometric features. Image sequence properties like scaling and unit are taken from **Dens Image**. A single channel of this sequence (if it is multichannel) must be selected with the buttons to the right of the parameter.

The measurement results can be stored to database files. These files are tab delimited ASCII files which can be easily imported to major Windows programs like text processing or spreat sheet application. Writing database files are independently supported for object and volume features. Activating the corresponding check boxes enables it. The name of the database is defined with the field **Database**. The files will be located in the subdirectory DATA of the main installation directory. The filename extension TXT will be added automatically.

If the check box **Label** is activated a single channel sequence will be generated. It contains all the measured objects, each object is coloured homogeneous but in different colours. To copy all measurement values to the clipboard activate the check box **Clipboard**.

A single object of interest can be visualized. Clicking on a specific row in the data grid chooses the object. By selecting a row in the data grid a new image is created with the object of interest visualized. The visualization depends on the settings in the **Object Visualisation** field. If **Render** is chosen, the object of interest is displayed with the **Surface Rendering** method. If **Mask** is chosen, the object is labelled in a pseudo colour in a new image stack.

Parameters:

Mask Image	Single channel mask image sequence that defines the objects
Dens Image	Image sequence for densitometric measurement and property source
Object	Stores measurement values of objects, including database filename
Volume	Stores volume measurement values of objects, including database filename
Label	Generates an image sequence with all objects labelled in different pseudo colours
Clipboard	Measurement values are automatically written to the clipboard